Barrier Function of the Follicle-Associated Epithelium in Stress and Crohn’s disease

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Även en tusenmilafärd börjar med ett steg

Kinesiskt ordspråk

Till Alpha och Mathilda
ABSTRACT

The earliest observable signs of Crohn’s disease are microscopic erosions in the follicle-associated epithelium (FAE) covering the Peyer’s patches. The FAE, which contains M cells, is specialised in sampling of luminal content and delivery to underlying immune cells. This sampling is crucial for induction of protective immune responses, but it also provides a route of entry for microorganisms into the mucosa. Crohn’s disease is associated with an increased immune response to bacteria, and the disease course can be altered by stress.

The overall aim of this thesis was to study the effects of stress on the FAE and elucidate the role of FAE in the development of intestinal inflammation, specifically Crohn’s disease.

Initially, rats were submitted to acute and chronic water avoidance stress to study the effects of psychological stress on the FAE. Stressed rats showed enhanced antigen and bacterial passage, and the passage was higher in FAE than in regular villus epithelium (VE). Further, stress gave rise to ultrastructural changes. Subsequent experiments revealed the stress-induced increase in permeability to be regulated by corticotropin-releasing hormone and mast cells. Furthermore, vasoactive intestinal peptide (VIP) mimicked the stress effects on permeability, and the VIP effects were inhibited by a mast cell stabiliser.

Human studies of ileal mucosa from patients with non-inflammatory disease and healthy controls showed a higher antigen and bacterial passage in FAE than in VE. In patients with Crohn’s disease, the bacterial passage across the FAE was significantly increased compared to non-inflammatory and inflammatory controls (ulcerative colitis). Furthermore, there was an enhanced uptake of bacteria into dendritic cells, and augmented TNF-α release in Crohn’s disease mucosa.

Taken together this thesis shows that stress can modulate the uptake of luminal antigens and bacteria via the FAE, through mechanisms involving CRH and mast cells. It further shows that human ileal FAE is functionally distinct from VE, and that Crohn’s disease patients exhibit enhanced FAE permeability compared to inflammatory and non-inflammatory controls.

This thesis presents novel insights into regulation of the FAE barrier, as well as into the pathophysiology of Crohn’s disease by demonstrating a previously unrecognised defect of the FAE barrier function in ileal Crohn’s disease.

Keywords: Corticotropin-releasing hormone, Crohn’s disease, \(^{51}\)Cr-EDTA, Escherichia coli, follicle-associated epithelium, horseradish peroxidase, human, ileum, inflammatory bowel disease, intestinal mucosa, mast cell, M cell, permeability, Peyer’s patches, rat, Ussing chamber, vasoactive intestinal peptide, villus epithelium
LIST OF PAPERS

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

I. Increased antigen and bacterial uptake in follicle-associated epithelium induced by chronic psychological stress in rats.

II. Characterization of antigen and bacterial transport in the follicle-associated epithelium of human ileum.

III: Increased uptake of non-pathogenic E. coli via the follicle-associated epithelium in ileal Crohn’s disease.

IV. Stress-induced barrier disruption of the follicle-associated epithelium involves corticotropin-releasing hormone, vasoactive intestinal peptide and mast cells.
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ABBREVIATIONS

$^{51}$Cr-EDTA  $^{51}$chromium-EDTA
CRH  corticotropin-releasing hormone
CRH-R  corticotropin-releasing hormone receptor
E. coli  *Escherichia coli*
FAE  follicle-associated epithelium
HRP  horseradish peroxidase
IBD  inflammatory bowel disease
Isc  short circuit current
M cell  membranous or microfold cell
NK-1R  neurokinin-receptor 1
PD  transepithelial potential difference
SED  subepithelial dome
TER  transepithelial electrical resistance
VE  villus epithelium
VIP  vasoactive intestinal peptide
VIPR  vasoactive intestinal peptide receptor
WAS  water avoidance stress

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1. INTRODUCTION

1.1. Crohn’s disease

1.1.1. History
Clinical descriptions of gastrointestinal disease resembling Crohn’s disease go back to the 16th century 1, when G. F. Hildenus noted during an autopsy of a boy suffering from abdominal pain and diarrhoea, that the ulcerated cecum was contracted and ivaginated into the ileum. Similar reports during the 16th-19th centuries indicated the appearance of a unique intestinal inflammatory disease that today would be identified as Crohn’s disease. Although Dalziel already published a paper in 1913 of a series of patients with granulomatous small bowel inflammation 2, it is the report by Crohn, Ginzburg and Oppenheimer at Mt Sinai Hospital in New York that is considered as the original description 3. In this classic paper, Crohn and his colleagues describe a condition of abdominal pain, emaciation, diarrhoea and fever. Originally, Crohn himself named the disease terminal ileitis since it was believed to be strictly localised to the small bowel. However, criticism was raised that the disease could also occur at other locations, and the name was changed to regional enteritis in the publication 3. Today it is well established that Crohn’s disease is a chronic episodic, inflammatory disease that can affect the entire gastrointestinal tract, from the mouth to the anus, however, the ileocaecal region of the bowel is most commonly affected.

1.1.2. Epidemiology and symptoms
Crohn’s disease is a Western world disease with the highest incidence rates in Scandinavia, Great Britain and North America 4. The disease has a slightly female predominance and onset at young adulthood with a peak incidence between 15-30 years. In 1991, the incidence in Sweden
was 6.1 in 100 000 per year and the prevalence was 146 in 100 000. Since this date, no further epidemiological studies in Sweden in general have been reported, however, a recent study showed that the incidence in Stockholm between 1990 and 2001 was 8.3 in 100 000 per year and the prevalence in the 1st of Jan 2002 was 213 in 100 000. Together with ulcerative colitis, Crohn’s disease constitutes the main condition of inflammatory bowel diseases (IBD).

The symptoms of Crohn’s disease are dependent on the location of the inflammation but abdominal pain, diarrhoea, weight loss, fever and vomiting are common features. The presence of abdominal and perianal fistulae are typical for the disease.

It is not fully understood how Crohn’s disease is initiated, however, studies have shown that the first observable signs of the disease are ileal aphaltoid lesions, well recognised by endoscopy. These lesions have shown to progress over time to larger ulcerations and stricturing of the lumen. Initially, it was observed that the lesions mainly occur at the lymphoid follicles. It has been shown that they can vary in size from barely visible to 3 mm in diameter. They are found in 70 % of the Crohn’s disease patients, and most commonly in the clusters of lymphoid follicles called the Peyer’s patches of the distal ileum. Further, magnifying endoscopy and scanning electron microscopy have been used to demonstrate that the aphaltoid lesions of Crohn’s disease are preceded by 150-200 μm sized ultra-structural erosions of the epithelium covering the Peyer’s patches, the so called follicle-associated epithelium (FAE). The early inflammation in Crohn’s disease is often located at the distal ileum, where Peyer’s patches are more frequent. Taken together, these observations suggest that the lymphoid follicles are the sites of initial inflammation in ileal Crohn’s disease, where the ulcerations originate.
from small erosions over the FAE. The FAE and Peyer’s patches are further discussed in paragraph 2.1.2.

1.1.3. General treatment
In the report from 1932, Crohn et al. proposed resection of the diseased segment as a cure, and for a long time, radical bowel resection was the only treatment. However, the development of anti-inflammatory drugs in the 1950’s, and increased knowledge about the disease as a panenteric disorder has lead to a more restrictive surgical approach.

The treatment of Crohn’s disease is unsatisfactory, since none of the existing treatments such as 5-aminosalicylates, corticosteroids, immunomodulators (e.g. azathioprine and methotrexate) or surgery, are curative. Although these treatments have a positive effect on most patients, the occurrence of relapse is high.

An example of a newer biological medication is infliximab (Remicade®) that induce remission of the disease by antibodies against TNF-α. Infliximab and other new immunomodulators are utilised with the goal of keeping the disease in remission and there is very little evidence that these treatments alter the natural history and disease course.

1.1.4. Aetiology
The exact cause of Crohn’s disease is unknown but evidence shows that genetic, immunological and environmental factors all contribute to the pathogenesis of the disease (Fig. 1).
Genetics

Epidemiological studies have shown that ethnic background and family history are of importance in the susceptibility of Crohn’s disease. First degree relatives of patients with Crohn’s disease show a 10 to 30 times increased risk of acquiring the disease. A number of studies have reported that 10-15% of first degree relatives have increased intestinal permeability in the absence of clinical symptoms, and approximately 30% have increased intestinal permeability compared to controls, after ingestion of acetylsalicylic acid. Furthermore, twin studies have demonstrated a higher pair concordance rate in monozygotic twins with Crohn’s disease, than in dizygotic twins.

In 2001, the first susceptibility gene for Crohn’s disease, CARD15/NOD2, was identified on chromosome 16 (IBD1), and since then, the results have been widely replicated. Whether the Crohn's-associated CARD15 mutations lead to a loss or gain of function of the NOD2 receptor is subject to controversy, and by which mechanisms this change in function might increase the susceptibility to Crohn’s disease is still under investigation. A recent study showed that high mucosal permeability in
healthy first degree relatives is associated with the presence of CARD15 3020insC mutation, indicating that genetic factors may be involved in impairment of intestinal barrier function in families with IBD. Since CARD15/NOD2 variants only seem to account for 10-30% of Crohn’s disease patients, several groups have focused on finding other candidate susceptibility genes associated with the disease. Additional putative loci have been mapped to chromosome 5 (OCTN gene), 6 (IBD3), 10 (DLG5 gene), 12 (IBD2), 14 (IBD4), and 19 (IBD6). The barrier defect seen in IBD share several pathophysiological and clinical characteristics with other barrier disorders and studies have revealed many disease-associated genes. For example, CARD15/NOD2 mutations have also been observed in Blau syndrome, and also in early onset of sarcoidosis, suggesting a role for the NOD2 gene in the development of granulomatous diseases, probably by inappropriate activation of the immune system. The association of NOD2 mutations has also been shown in allergic diseases and atopy, which might indicate that NOD2 also plays a role in the Th2 pathway.

Aside from the genetic studies in humans, several animal models have been developed to map the genes involved in the aetiology of IBD. The overall lesson from animal studies is that genetics alone is not enough to cause Crohn’s disease, but an interaction with alterations in the microflora, epithelial barrier, immune response, enteric nerves and other components of the intestine, can contribute to the development of the disease.

**Immunology**

Under normal conditions the immune system acquires tolerance towards luminal antigens. The tolerance is induced by regulatory T-cells, and/or anergy or deletion of antigen specific T-cells. This phenomenon is called oral tolerance. It has been found that T-cells from patients with Crohn’s
disease, in contrast to the normal population, produce cytokines in response to dietary antigens and the patients’ own microfloral antigens. This suggests that patients with Crohn’s disease have impaired oral tolerance, possible caused by a defect barrier. Moreover, studies show a disturbed IgA-production, with a reduced in vivo secretion of IgA in Crohn’s disease. Large numbers of immunoglobulin producing cells in the lamina propria have been observed and in inflamed mucosa increased numbers of T-cells, mast cells, and macrophages are seen together with inflammatory mediators like prostaglandin E2, leukotriene B4, histamine, substance P and nitric oxide. Crohn’s disease is thought to be of Th1 type inflammation, as shown by increased production of cytokines important in cell-mediated immunity such as IL-2, IL-6, IL-1β, IFN-γ and TNF-α, and, in inflamed mucosa, HLA class II molecules on the epithelial cells. Though, in early stages of Crohn’s disease, increased levels of the Th2 cytokine IL-4 have been found.

Environment

Microflora

Luminal enteric bacteria are the most important inflammation-driving environmental factor in Crohn’s disease. Patients with Crohn’s disease have an increased number of adherent-invasive Escherichia (E.) coli in the mucosa, and the concentration of bacteria increases progressively with the severity of the disease. Although no specific pathogen has been proven as a causative factor, several bacteria have been linked to Crohn’s disease, for example Mycobacterium paratuberculosis and Yersinia pseudotuberculosis, and the role of the luminal microflora is evident. There are some animal models present where inflammation is caused by microbial infection, such as models of infectious murine colitis. Studies in these models have helped to define the interactions between bacterial pathogens and host defence. For
instance it has been shown that rats susceptible to intestinal inflammation do not develop IBD when bred under germfree conditions 49. Aside from being inflammation-driving, bacteria can also be protective, and probiotics like bifidobacteria and lactobacilli are thought to protect against IBD 50.

Diet
The influence of food in Crohn’s disease aetiology has been widely investigated in the Western world. It has been shown that dietary factors may have effect on disease activity 51, and an enhanced intake of fast food, fat and refined sugar, and reduced intake of fresh fruit are important risk factors in the development of Crohn’s disease 4. Moreover, elemental diet can be used for inducing remission in the disease and for nutrition before a bowel resection 52,53. An increasing incidence of Crohn’s disease has been found in Asia 6,54, formerly a low-incidence part of the world. A probable cause could be the influence of Western style, such as food.

Smoking
In several studies it has been shown that a higher degree of patients with Crohn’s disease are smokers compared to controls 55. Although a few studies have shown that smoking has no negative effect on the development of Crohn’s disease 56-58, it is generally believed that smoking more than doubles the risk of acquiring the disease 59,60 and that patients with Crohn’s disease that smoke are associated with greater disease activity and higher surgical rates 61,62.
Stress

Environmental stress has been shown to alter the course of Crohn’s disease 63;64. Stress and its effects on gastrointestinal disease are further described in paragraph 2.5.2.

Barrier function

A disturbed intestinal barrier function has been suggested as a factor for Crohn’s disease 65;66. Normally, only small amounts of protein antigens cross the epithelium and interact with the immune system. However, in Crohn’s disease there is an increased small bowel permeability to medium sized probes and antigens 65;67;68, leading to increased antigen exposure to immune cells, that in turn can contribute to inflammation and gastrointestinal disease 37;69;71. Several studies have shown an increased permeability also in healthy relatives of patients with Crohn’s disease 18;20;21, and both patients with Crohn’s disease, and their relatives, have demonstrated an increased intestinal permeability when exposed to NSAIDs 19;21;72. Moreover, studies have reported a high prevalence of increased permeability in spouses of patients with Crohn’s disease 20;21;73, suggesting that environmental factors are of importance in the development of the disease. Though, recently a very extensive study showed that a common environment with Crohn’s disease patients was not associated with increased permeability in family members 27.

Crohn’s disease has been suggested as a tight junction disorder 74. Structural changes 75 and leaky of the tight junctions in response to luminal stimuli 76 has been demonstrated in Crohn’s disease mucosa. Another factor in Crohn’s disease is mucus. It has been shown that inflamed colon mucosa of Crohn’s disease patients has a thicker mucus layer 77 and an altered structure of mucus glycoproteins 78;79. This might result in less protective mucus layer, despite its increased thickness.
Since patients with Crohn’s disease have shown an enhanced permeability, and the barrier function of FAE has not previously been studied, studies regarding environment, barrier function and immune system are of importance to elucidate the initial steps of the pathogenesis of Crohn’s disease.
2. **BACKGROUND TO THE STUDY**

2.1. **Structure and function of the small intestine**

The human small intestine is approximately 4-5 m long and is divided into duodenum, jejunum and ileum, where the ileum is the most distal part. The total surface area of the intestinal epithelium is 3-400 square meters. From the stomach to the rectum, the inner surface is covered by a single-cell polarised epithelial layer that digests and absorbs nutrients at the same time as it constitutes a barrier between the inner and outer milieu.

The epithelium that lines the mucosal surfaces of the small bowel consists of villus epithelium (VE) and FAE ⁸⁰ (Fig. 2). The exact distribution of FAE and VE in the human intestine is not known. The entire FAE presents a biochemical face to the lumen that is distinct from the surrounding VE ⁸¹. While the VE is specialised for digestion and absorption of nutrients, the FAE is specialised in antigen sampling.

![Diagram of villus epithelium (VE) and follicle-associated epithelium (FAE) of the ileum.](image)

*Fig. 2. Villus epithelium (VE) and follicle-associated epithelium (FAE) of the ileum.*
2.1.1. The small intestinal wall covered by VE

The small intestinal wall lined by VE consists of three main tissue structures; mucosa, submucosa and muscle layers (Fig. 3). The small bowel has digestive, absorptive, secretory and immunological functions that mainly take place in the mucosal layer.

![Diagram of the small intestinal wall covered by villus epithelium.]

**Fig. 3. Schematic illustration of the small intestinal wall covered by villus epithelium.**

The mucosa can be further divided into epithelium, lamina propria and muscularis mucosae. The epithelium is organised in fingerlike villi, which project into the lumen, and crypts, that extend down into the basal layer and often reach the muscularis mucosae. The VE surface area is increased by submucosal foldings, so called plicae circulares, and the covering of the villi by tightly packed microvilli. The epithelial cells are connected laterally to each other by junctional complexes (see paragraph 2.3.3), and are separated from the underlying lamina propria by a thin basement
membrane, basal lamina, which is mainly composed of collagens, laminins, and proteoglycans (Fig. 3). The lamina propria lies beneath the epithelium and consists of loose connective tissue forming the core of the villi and surrounds the crypts. The most abundant cell types in lamina propria are mononuclear immunocompetent cells like plasma cells, lymphocytes, and macrophages, but, eosinophils, mast cells, fibroblasts, myofibroblasts, smooth muscle cells also occur. Underneath the lamina propria is the muscularis mucosae which is a thin sheet of smooth muscle cells bordering the underlying submucosa. The physiological role of muscularis mucosae is unclear, but it is thought that it may contribute to the movement of villi and emptying of luminal contents of the crypts. The submocosa is a more densely collageneous, less cellular structure than the mucosa. Major blood vessels, lymphatics, nerves, ganglia, and occasionally lymphoid collections are located here. The muscle layer consists of the muscularis propria, constituting of the inner circular and the outer longitudinal muscle layer, and the serosa consisting of loose, connective tissue with fat, collagen and elastic tissue.

Cell types in VE
As the major function of the small bowel epithelium is nutrient absorption, enterocytes constitute 85 % of the cells lining the villi. The absorptive capacity of the enterocytes is 20-40 times increased by the lining of a brush border membrane constituting of closely packed microvilli on the enterocytes surface. Anchored to the brush border is the glycocalyx which protects the epithelium and prevents uptake of antigens and pathogens. It mainly constitutes of large carbohydrates, but also enzymes and proteins essential in digestion and absorption. Both the crypts and villi consist to 20 % of the mucin-producing goblet cells. The mucins are glycosylated proteins constituting the main part of
the mucus that protects the epithelial surface throughout the intestine. The exact regulation of goblet cells is unclear. About 3.5% of the epithelial cells are found to be Paneth cells. Located at the base of the crypts they prevent microorganism proliferation by a variety of secretory granules enrolling for example lysozyme, TNF, phospholipases, and antimicrobial peptides called defensins \(^{83}\). Enteroendocrine cells are spread throughout the epithelium, and in response to changes in the external microenvironment, or signalling from enteric nerves, they release gastrointestinal hormones like secretin, neurotensin and somatostatin. Remaining cells of the epithelium include the so-called cup cells that are thought to have an affinity for distinctive bacterial pathogens, and the tuft cells whose role is unknown.

The ability of VE to face the outer environment is enhanced by the noncellular defenses produced by epithelial cells. These are among others, mucins, defensins, and secretory antibodies. The most important antibody in mucosal surface protection is IgA, that is produced by plasma cells in the lamina propria and then transported to the apical surface and secreted into the gut lumen where they bind to the pathogens, thus limiting their adherence and colonisation \(^{84}\).

2.1.2. The Peyer’s patches covered by FAE

The small intestinal wall lined by FAE constitutes of clusters of organised lymphoid structures that are spread throughout the human intestine \(^{85}\). It was more than 300 years ago that J.K Peyer described these aggregations of lymphoid cells in the small intestinal wall, and named them “folliculi lymphatici aggregate”, consequently there has to be more than one follicle to form a Peyer’s patch. Peyer’s patches are found in all parts of the small bowel. In humans, they develop well before birth, though the
full development of the patches as inductive sites requires acquired antigenic challenge 86. In adolescence more than 240 patches are found in the small intestine, however, the number regresses with age and in 90-year olds, the number of observable patches is around 50 87. In 2002, Van Kruiningen et al described the anatomic distribution of the Peyer’s patches in humans 12. By using distal ileum, obtained at autopsy from 55 adults without intestinal disease, the number, location and size of patches were recorded. The mean number of patches in the distal ileum was evaluated to 24 (range 19-30), however, the number of patches turned out to vary between individuals. In addition, it was revealed that also the size of a single patch varies between individuals, with the largest patches, approximately 50 cm², in 21-30 years-old compared to approximately 30 cm² in younger and older individuals. The variation in distribution, size and numbers of the human Peyer’s patches have been demonstrated previously 87.

The Peyer’s patches consist of numerous follicles separated from each other by interfollicular zones, characterised by high endothelial venules (HEVs) surrounded by densely packed lymphocytes, mainly T cells, but also dendritic cells 88 (Fig. 4.). The follicles consist of B-cell germinal centres and a marginal zone constituting of proliferating B lymphocytes expressing IgM and IgG, and phagocytotic macrophages. Between the FAE and the follicle, a specialised tissue called the dome area or the subepithelial dome (SED) is located. The SED constitutes of T cells and B cells, and is rich in phagocytizing dendritic cells, macrophages and monocytes. It has been proposed that luminal antigens and pathogens sampled by the FAE are further captured by immature dendritic cells within the SED and ferried to adjacent interfollicular T cells where dendritic cell maturation and antigen presentation would occur 89.
After initiation of an immune reaction, primed B lymphocytes in Peyer’s patches preferentially migrate as precursors of IgA secreting plasma cells via the lymphatics, mesenteric lymph nodes and peripheral blood to the lamina propria. Because of this homing of lymphocytes, the term “gut associated lymphoid tissue” or GALT was introduced. In following studies it was shown that the lymphoblasts also migrated into other organs lined with mucosal membranes, which gave rise to the term “mucosa-associated lymphoid tissue” or MALT.

**Cell types in FAE**

The entire FAE presents a surface to the lumen that is different from the surrounding VE. As in VE, enterocytes are the most common cell, and as their counterparts in VE they have a complex network of glycocalyx, but they are not identical to VE enterocytes. For example, they express lower amounts of the membrane-associated hydrolases involved in digestive functions, and the glycosylation patterns differ from those in
VE enterocytes \(^{80;90;91}\). These features together facilitate the recognition and adherence of microorganisms to the FAE.

The number of Paneth cells in the FAE crypts is decreased and there are less goblet and enteroendocrine cells. In addition, the FAE lacks the subepithelial myofibroblast sheath, and the basal lamina is more porous compared with that in VE \(^{92}\).

Moreover, the entire FAE lacks polymeric IgA receptors and therefore it is unable to transport protective IgA to the lumen \(^{80}\). These characteristics together promote local contact of intact antigens and pathogens with the FAE surface.

In contrast to VE, the FAE contains so called *membranous or microfold (M)* cells, specialised in antigen sampling and transport \(^{88}\) (Fig. 5).

**Fig. 5.** Photograph and schematic illustration of an M cell (M) between to enterocytes (E) in the follicle-associated epithelium. Internalised antigen (arrows) and bacteria are transported in vesicles across the M cell and delivered to immune cells (T and B lymphocytes, macrophages and dendritic cells) in the M cell pocket and underlying subepithelial dome. L = lymphocyte.
In humans and rats it is known that approximately 10 % of the FAE constitutes of M cells. M cells differ in morphology from the adjacent FAE enterocytes. The apical membrane has microfolds, or ruffles, rather than microvilli and are further characterised by the lack of an organised brush border, short and irregular microvilli, and reduced expression of digestive enzymes, such as sucrase, isomaltase and alkaline phosphatase. They have a high endocytotic activity of adherent, as well as fluid-phase macromolecules and particles, and have very few lysosomes. The basolateral plasma membrane is deeply invaginated and forms pockets containing T and B lymphocytes in addition to professional antigen-presenting cells. These pockets decrease the travelling distance for the endocytotic vesicles, from the apical to the basolateral side, ensuring rapid and efficient transcytosis.

There are several different theories concerning the origin of M cells. The first one is that they are generated from the same pluripotent stem cells as enterocytes, Paneth cells and goblet cells, and this theory is supported by a recent study showing the presence of M cells in regular VE. The second theory is that M cells are formed directly from the enterocytes under the influence of B lymphocytes or bacteria in vivo. In contrast, a third group of scientists believe that M cells are formed in specific FAE-associated crypts when stimulated with lymphocyte-derived factors. Finally, the last theory is that M cells are of clonal origin and represent a separate cell line.

Unfortunately, the histochemical properties of FAE and M cells vary according to species and location. For example, markers have been identified for pig, rabbit and mouse, however, there is today no reliable rat or human M cell marker, which hampers the possibility to elucidate the actual role of M cells in barrier function of the FAE in rats and humans.
**Bacterial uptake via FAE and M cells**

The key features of FAE that face the lumen is the facilitation of the uptake of antigens and various microorganisms such as bacteria, viruses and protozoa\textsuperscript{108}. Several microorganisms, particularly bacteria, take advantage of the transcytotic function of the M cells and use them to cross the otherwise impermeable epithelial lining of the gut. Both in experimental animal models, and when cultured \textit{in vitro} together with human intestinal biopsies, strains of \textit{E. coli}, \textit{Yersinia}, \textit{Salmonella} and \textit{Shigella} exhibit specific adherence to FAE and M cells\textsuperscript{109;110}.

The adhesion of enterohaemorrhagic \textit{E. coli} (EHEC) and enteropathogenic \textit{E. coli} (EPEC) to FAE is characterised by intimin binding to the translocated intimin receptor, which is exported by the bacteria and integrated into the host cell plasma membrane\textsuperscript{109;110}. Studies have shown that \textit{Yersinia enterocolitica} utilises the FAE and M cells as entry sites via specific binding of invasin to β1-integrin on the surface\textsuperscript{111;112}. In mouse FAE, β1-integrin is specifically expressed on M cell surface\textsuperscript{112}, and in a human model FAE, we recently showed, increased β1-integrin expression in FAE compared to VE\textsuperscript{113}.

\textit{Salmonella typhimurium} crosses the epithelial barrier by attaching to either M cells or enterocytes\textsuperscript{109}. Subsequent events include M cell/enterocyte destruction and subepithelial migration of macrophages.

\textit{Shigella flexneri} uses the M cells for its initial entry and once across the luminal surface, the bacteria may invade adjacent enterocytes\textsuperscript{109}. \textit{Shigella flexneri} has shown to direct its own uptake into rectal and colonic mucosa through membrane ruffling and secretion of effector proteins that induce endocytosis of \textit{Shigella} by colonic M cells. After crossing the M cell, the bacteria are engulfed by macrophages leading to the induction of inflammatory responses.
2.3. Intestinal barrier function

The intestinal mucosa is continuously exposed to a heavy load of antigenic molecules from ingested food, resident/invading bacteria and viruses. The ability to control invasion of harmful substances from lumen is defined as intestinal barrier function. The exact mechanisms involved are as yet not fully elucidated but barrier function can be divided into four components. The importance of the barrier components described depends on the chemical, physical and immunological nature of the luminal contents.

The first component is the lumen itself, where bacteria and antigens are degraded by gastric acid, pancreatic and biliary juices. The second component is the microclimate constituting of the unstirred water layer, glycocalyx, IgA, and the mucus layer, that prevents adhesion of pathogenic bacteria to the epithelium by mucin-binding sites that compete with the epithelial binding sites, thus impeding bacterial-epithelial interaction. The third component is the epithelium with chloride secretion, basal lamina and epithelial cells, connected to each other via junctional complexes. Within the epithelium there are numerous antimicrobial peptides whose function is to kill microorganisms, attract monocytes, and potentiate macrophage opsonisation. One family of antimicrobial peptides is the defensins, which can be found in the Paneth cells of the crypts of the small bowel (α-defensins) and throughout the colonic epithelium (β-defensins). Finally, lamina propria make up the last barrier component and consists of immunoglobulins, cells of acquired and innate immunity, the enteric nervous system, hormones and the endothelium of the capillaries. In addition, intestinal propulsive motility, rapid repair process and cell turnover also are involved in barrier function 114. If the control of the barrier function is disturbed, it can lead to enhanced antigen and bacterial passage which in turn may damage the mucosa leading to pathological conditions 115.
2.3.1 Permeability

Intestinal permeability is defined as the non-mediated intestinal passage of medium-sized hydrophilic molecules, i.e. passage of molecules down a concentration gradient without the assistance of a carrier system. When measuring intestinal permeability it is necessary to use simplifications, since the definition permeability refers to the passage of a solute through a simple membrane, whereas the intestinal membrane consists of several layers and different cell types.

Several clinical disorders are believed to result from altered permeability, induced by loss of mucosal integrity. The most common ones are IBD, celiac disease, intestinal ischemia, food intolerance, rheumatoid arthritis, allergy and malnutrition. Moreover, mucosal integrity can also be affected by treatment with acetylsalicylic acids 120,121.

In vivo, intestinal permeability is usually measured as the permeability to paracellular markers that are taken up via the junctional complex. In in vitro systems, intestinal permeability is often measured as the transepithelial electrical resistance (TER) which correlates with the ability for passive diffusion of ionic charge across the epithelia 122.

2.3.2 Uptake and transport

There are several ways for solutes to cross the intestinal epithelium (Fig. 6). Lipid soluble or small hydrophilic compounds pass through the cells via passive diffusion into the lipid bilayers, while larger hydrophilic molecules pass via the tight junctions and intercellular spaces in the paracellular route. The paracellular route, via the junctional complexes, is believed to be impermeable to protein-sized molecules and thus, under normal conditions, it constitutes an effective barrier to antigenic macromolecules. A controlled protein uptake by the intestinal mucosa is physiologic and essential for antigen surveillance in the gastrointestinal
tract. Small hydrophilic molecules can also pass the barrier transcellularly via aqueous pores.

Fig. 6. Schematic illustration of passage routes across the epithelium. (A) Transcellular route (lipophilic and small hydrophilic compounds). (B) Paracellular route (larger hydrophilic compounds). (C) Transcellular route via aqueous pores (small hydrophilic compounds). (D) Active carrier-mediated absorption (nutrients). (E) Endocytosis, followed by transcytosis and exocytosis (larger peptides, proteins and particles).

In addition to the non-mediated permeation routes described, there are two active ways to cross the epithelium. First, there are numerous carrier-mediated mechanisms for uptake, utilised for sugars, amino acids and vitamins, and second, larger peptides, proteins and particles may be endocytosed into endosomal vesicles and transported through the cells via transcytosis.
2.3.3. The junctional complex

The junctional complex was first described in 1963 when Farquhar and Palade showed that enterocytes were attached to each other via tight junctions, adherens junctions, desmosomes and gap junctions (Fig. 7).

![Diagram of the junctional complex]

Fig. 7. The junctional complex.

Tight junctions, also called zonula occludens (ZO) are located at the apical part of the lateral membrane forming a network of linking strands (Fig. 8). They are important in epithelial transport towards and away from the lumen (gate function), as well as in maintaining the polarity of lipid substances by preventing diffusion from the apical to the basolateral region in the outer cell membrane (fence function). These functions seem to be regulated in different ways. The gate function is important in the intestinal barrier regulation and has been shown to be affected by many intracellular second messengers such as cAMP, Ca\(^{2+}\), phospholipase C, protein kinase C, calmodulin and G-proteins. There is a size and charge-selectivity within the tight junction permeability barrier, where positively charged molecules and ions pass
more easily. Tight junctions appear as focal contacts ("kisses") on the plasma membrane. These contacts correspond to continuous fibrils, where fibrils on one cell interact with fibrils on the adjacent cell. The fibrils are formed by at least two types of transmembrane proteins, occludin and different variants of the claudin family.

The discovery of claudins in 1998 significantly advanced the understanding of the tight junction barrier. The human claudin family includes at least 24 members and the distribution of them varies in different tissues, which probably explains the variable permeability seen in diverse tissues. Occludin and claudins are connected to protein complexes called cytoplasmic plaques. These constitute of ZO-1, ZO-2,
ZO-3, cingulin, 7H6 antigen, and several other proteins with unidentified function including symplekin, ZA-1TJ, p130 and ZAK. The cytoplasmic plaques are further linked to F-actin filaments of the cytoskeleton. The exact way in which F-actin is linked to the tight junctions is unknown, but strong evidence show that the paracellular permeability is influenced by the state of perijunctional actin.

Interestingly, a recent study showed that actin-depolymerising drugs caused disruption of the tight junctions by removing occludin via endocytosis. Although this observation does not elucidate the functional role of occludin, or the detailed mechanisms of actin depolymerisation-induced tight junction disruption, it does suggest an important role for occludin endocytosis in the regulation of tight junctions.

Alterations of tight junction structure and/or function have been found in several disease states, and structural changes have, for example, been found in inflamed mucosa of Crohn’s and celiac disease patients. Several inflammatory mediators involved in inflammatory diseases have been shown to affect tight junction permeability, such as nitric oxide, and the inflammatory cytokines INF-γ, TNF-α, and IL-4. Some pathogens also have the ability to disrupt the intestinal barrier via the tight junctions, for example zonula occludens toxine (ZOT) from Vibrio cholerae and the toxin A derived from Clostridium difficile. Moreover, abnormalities in ZO-1 localisation have been found in epithelia infected with Salmonella typhimurium.

Adherens junctions, located below the tight junctions, are actin- and myosin-associated membrane structures formed by adhesion molecules of the cadherin family and their cytoplasmatic binding proteins α-, β-,
and γ-catenin. It has been suggested that adherens junctions together with tight junctions form one single functional unit 146.

*Desmosomes* form spot-like dense adhesions between the epithelial cells and are connected to the intermediate filaments of the cytoskeleton. Desmosomes are dispersed all over the lateral cell surfaces, however, they are frequently found to be concentrated below the adherens junctions.

*Gap junctions* are arrangements of cylindrical tubuli consisting of proteins called connexins. Gap junctions function as intercellular channels allowing ions and small molecules to pass between cells, thus linking the interior of adjacent cells.

### 2.3.4. Endocytosis

Endocytosis is defined as uptake of extracellular particles and molecules into the cells by invagination of the plasma membrane and vesicle formation. Endocytosis in epithelial cells can occur in different ways, depending on the nature of the substance that is taken up (Fig. 9).

*Fig. 9.* Uptake of extracellular material via different types of endocytosis.

R = receptor.
Endocytosis occurs in enterocytes of both VE and FAE, however, it is well known that endocytosis of bacteria and particles primarily occur via the M cells in the FAE 147.

The first route, present in both enterocytes and M cells, is via clathrin-mediated endocytosis 148-150, a highly specific receptor-mediated process, utilised mainly by immunoglobulins, viruses and growth factors from breast milk. The clathrin-coated vesicles seldom become larger than 150 nm in diameter 151. In this special type of endocytosis the cells synthesise receptors and internalise molecules that have bound specifically to them 152.

Larger (up to several μm in size) bacteria, viruses, and particles are taken up via an adsorptive endocytosis, or phagocytosis 153, involving binding of molecules to the cell membrane via receptors. Phagocytosis is relevant for the non-specific uptake of luminal dietary and bacterial antigens, and the process is triggered by secreted solubles from the invading bacterium 154. Phagocytosis is a more common process in M cells than in enterocytes 109;155-157.

Both enterocytes and M cells are capable of actin-dependent non-specific fluid-phase endocytosis, or macropinocytosis, where substances in the luminal fluid are internalised 153. The process resembles phagocytosis, but is not receptor-mediated 152. For example, the protein antigen horse-radish peroxidase (HRP) is known to be taken up via macropinocytosis, preferently via M cells 158;159, but the exact way how HRP is sorted and transported after endocytosis is not fully elucidated.

In recent years, attention has been paid to a fourth mechanism, referred to as lipid rafts / caveolae. This endocytotic event involves a flask-shaped
invagination of cholesterol-enriched microdomains within the plasma membrane that may contain a coat protein, caveolin\textsuperscript{160}. Endocytosis via lipid rafts / caveolae is most common in endothelial cells but occurs also in enterocytes, although this type of endocytosis is rare in M cells as they contain few to no caveolae\textsuperscript{161}. Studies have shown that for example certain enterotoxins and viruses are endocytosed via rafts / caveolae. In addition, the endocytosis of occludin discussed in paragraph 2.3.3 has shown to occur via caveolae-mediated endocytosis\textsuperscript{135}.

2.3.5. Transcytosis
Following endocytosis, uptaken molecules must be transported through the cells via transcytosis. For this, enterocytes and M cells have different systems.

Enterocytes
Enterocytes have apical and basolateral sorting compartments, so called “apical early endosomes” and “basolateral early endosomes”, that share a “common recycling compartment”\textsuperscript{162}. These compartments are used during clathrin-mediated endocytosis and phagocytosis. In enterocytes, transcytosis can occur in three ways.

1) When vesicles bud off from the apical membrane, they can merge with the apical early endosomes and then be recycled back to the apical membrane, with or without cytoplasmic release of their content. The content (protein, virus or particle) bound to the internalised receptor is most often released into the cytoplasm upon acidification of the vesicles, while the receptor is delivered back to the cell membrane. However, large molecules like peptides and proteins may be degraded on their way to the basolateral membrane since they diffuse rather slowly through the cytoplasm.
2) The vesicle can join with the common recycling compartment and from there, the content (protein, virus or particle) is often directed into pathways leading to lysosomal degradation in lysosomes.

3) The vesicle can be transported from the apical to the basolateral side for subsequent merging with vesicles from the basolateral early endosomes, although this is a quite rare process compared to the others described 152;162;163.

Transport of intact proteins or carrier-mediated systems across enterocytes is not easy to achieve, however, studies have demonstrated that the endosomal sorting mechanisms can be modified in order to decrease apical vesicle recycling, thereby increasing the transcytosis of proteins across the epithelium 164.

It is known that enterocytes not only transport internalised antigen, in addition they can, during chronic inflammatory diseases such as IBD and celiac disease, act as non-professional antigen-presenting cells and promote inflammation 165-167.

**M cells**

Endocytosis via the FAE and M cells is well characterised, however, the transcytosis and fate of internalised content have not been very well studied 168. M cells only contain few lysosomes 156 and can not express MHC-II, consequently they can not function as true antigen-presenting cells 166. It is known that the apical part of the M cell cytoplasm contains several endosomes and vesicles with lysosomal markers on the surface 149;156. Ultrastructural studies have demonstrated that soluble tracer proteins infused into the lumen are incorporated into the membrane vesicles of the M cells and rapidly transported across the narrow bridge
of the apical cytoplasm, and released by exocytosis into the sequestered intraepithelial space \(^{158}\).

2.3.6. Regulation of endocytosis and transcytosis

Both endocytosis and transcytosis can be influenced by numerous factors. Although the mechanisms are not fully elucidated, bacterial exposure in one way or another leads to enhanced uptake and transport across the intestinal epithelium \(^{169}\). Bacterial stimulation also leads to the production of pro-inflammatory cytokines, increasing endocytosis and transcytosis. For example, TNF-\(\alpha\) has shown to induce HRP endocytosis in intestinal epithelial cell culture \(^{170;171}\), and increased transcytosis of HRP could be correlated to TNF-\(\alpha\) mRNA levels in the underlying mucosal tissue \(^{170}\). Another factor affecting epithelial uptake is intestinal disease, in which dysfunctional intestinal motility can prolong the exposure time to luminal bacteria. Furthermore, studies have shown that antigen-binding speeds up the transcytosis. For example, when conjugating HRP to IgE, the protein was carried across the epithelial membrane into the lamina propria within three minutes compared with hours for unconjugated HRP \(^{165}\).

In FAE, the size and number of Peyer’s patches and M cells are of importance for endocytosis and subsequent transcytosis. Smith et al reported that the number of M cells increased after transfer of germ-free mice to normal housing conditions \(^{172}\). Subsequently, several other studies have shown an increased number of M cells and enhanced particle uptake after bacterial stimulation \(^{99;101;173;174}\). However, Gebert et al \(^{169}\) recently found that the enhanced uptake seen after bacterial stimulation depends on increased transport capacity of the M cells already present in the FAE, and not an increase in numbers. In addition, intestinal inflammation may increase the M cell numbers. For example, indomethacin-induced ileitis in rats increases the M cell number and
apoptosis, which may alter the intestinal barrier function. Moreover, an increased number of M cells has been found in ileal mucosa of patients with spondylarthropathy, and the observation of M cell cytoplasm disruption, with lymphocytes entering the gut lumen at these sites, suggest a possible mechanism for the development of aphtoid ulcers.

2.4. Studies of intestinal permeability

2.4.1. In vivo
Intestinal permeability in vivo in humans was first studied using intestinal infusion of solutes. Today, intestinal barrier function is mainly studied as the urinary or blood excretion of orally ingested markers. Obviously, the characteristics of the permeability probes are of high importance and the ideal probe should be water soluble, non-toxic, nondegradable, and not be metabolised. Moreover, the probe should ensure complete urinary excretion and the analysis should be sensitive, accurate and uncomplicated. There is, of course, no probe that fulfils all these criteria, but some probes are close and consequently used for permeability studies. The most commonly used small pore markers (5-8 Å) are monosaccharides (mannitol, rhamnose), and polyethylene glycols (PEG), with molecular weight around 400 Da. The most frequently used large pore markers (9.5-11 Å) are disaccharides (lactulose, cellobiose), $^{51}$chromium-EDTA ($^{51}$Cr-EDTA), and PEG, with molecular weight around 1000 Da. Permeability is usually presented as the ratio between the large pore and small pore marker.

2.4.2. In vitro
The current in vivo methods for permeability studies of the human intestinal mucosa cannot elucidate passage routes and mechanisms involved in barrier function in IBD. In addition, there are considerable
difficulties with \textit{in vivo} studies of intestinal uptake of intact protein \textsuperscript{177}. \textit{In vitro} techniques offer possibilities to study processes in human tissue that would be impossible to study \textit{in vivo}, and much of the basic knowledge of gastrointestinal physiology has been achieved through \textit{in vitro} techniques. Several techniques for \textit{in vitro} studies of intestinal mucosa have been developed, and one of them is mucosal sheets in Ussing chambers \textsuperscript{178,179}. The good viability-supporting possibilities with oxygenation and effective circulation of the fluid on both sides of the tissue, combined with the possibility to monitor membrane electrophysiological parameters, provide the Ussing chamber technique with important advantages compared to other \textit{in vitro} techniques for intestinal tissue experiments \textsuperscript{180}.

\subsection*{2.4.3. The Ussing chamber}

The Ussing chamber was first described in 1951 by the Danish physiologists Ussing and Zerhan \textsuperscript{181}. The Ussing chamber technique has many applications, but mostly it is used to study ion transport, drug absorption, protein absorption, and studies of several pathophysiological processes in both animals and humans \textsuperscript{179,180,182,183}. The initial methodology of the Ussing chambers, that was rather complicated, was modified and simplified by Grass et al in 1988 \textsuperscript{184}. The principle is that a flat sheet of mucosa is mounted between two half-chambers filled with continuously oxygenated buffer (Fig. 10). The arrangement of the gas ports provide buffer circulation, which gives efficient mixing of the fluid and reduces the thickness of the unstirred water layer to physiological levels \textsuperscript{185}. The chambers are kept at 37°C and two pairs of electrodes enable the monitoring of electrophysiological parameters during the experiment. The marker solution is added to the mucosal buffer, and at defined time intervals samples are redrawn from the serosal buffer as a measurement of passage.
Fig. 10. The Ussing chamber. (A-B) Schematic drawing and photograph of the Ussing chamber. Tissue is mounted between the two chamber halves and is continuously oxygenated. One pair of Ag/AgCl-electrodes enables measurement of potential difference and one pair of platinum-electrodes gives current to the system. (C) Tissue is carefully mounted so it covers the entire surface area of the opening that connects the two chamber halves. (D) After mounting, chambers are filled with buffer and put in the 37°C Ussing chamber system.

The preparation techniques of intestinal tissue for the use in Ussing chambers depend on the species and bowel segment being studied\textsuperscript{180}. Generally, the intestinal specimens are dissected and immediately transported to the laboratory, in oxygenated buffer without circulation. Tissues are either unstripped, or stripped, which means dissection of the muscle layers. In unstripped bowel, the whole bowel wall is intact, and it is possible to evaluate effects of the enteric nerves on intestinal tissue. However, unstripped segments are not that often used in permeability studies because of the longer diffusion distance for the probe molecules. In stripped tissues, the external muscle layers and myenteric plexus are removed and the mucosa mounted thus consists of the epithelium, the underlying lamina propria and the muscularis mucosae. After mounting
in the chambers, tissues are equilibrated for 20-60 minutes to achieve steady state conditions in transepithelial potential difference (PD).

**Electrophysiology**

A characteristic for all transporting epithelia is the ability to maintain a PD. The ability depends on all the electrogenic ion pumps activity in the epithelial cell membrane, mainly Na⁺ / K⁺-ATPase, and on the epithelial barrier function \(^{186}\). Theoretically these two can be separated into the short circuit current (Isc) and TER. The Isc represents the current needed to nullify the PD and is a function of the ion pumps activity. The TER reflects the electrical resistance of the paracellular routes, mainly via the tight junctions. The electrodes enabling the monitoring of electrophysiological parameters during the experiments consist of one pair of Ag / AgCl-electrodes with agar-salt bridges and one pair of current-giving platinum electrodes. Since active ion transport requires energy production, the basal PD or Isc can be used as a measure of tissue viability. By passing the current (I) through the epithelium, the change in PD can determine the TER by Ohm’s law, \(PD = I \times R\). However, this calculation relies on a simplified epithelial model, viewing the epithelium as a parallel circuit consisting of paracellular and transcellular pathways.

2.5. Stress

2.5.1. The stress concept

Stress is a normal part of life and has been defined in many different ways \(^{187}\), for example as “any threat to the homeostasis of an organism” \(^{188}\) or “the range of tensions of modern life” \(^{189}\). An adequate stress response is essential for survival, but the ways of coping with stress is highly individual \(^{190}\). Under normal circumstances, physiological systems are turned on and off in response to stress, matching the duration and
severity of the stressors, a so-called adaptive response. However, in some individuals stress may become harmful and cause damage to the organism, a so-called maladaptive response, also referred to as pathologic stress. Maladaptive responses are often associated with chronic daily life stressors such as losses, financial problems, unemployment, and have been linked to exacerbations of irritable bowel syndrome symptoms.\textsuperscript{191}

Regardless if the threat is real (physical), perceived (physiological) or environmental, the principal stress responses triggered to maintain homeostasis are quite similar.\textsuperscript{192} Normally, the stress response constitutes of a behavioural response (e.g. anxiety), an autonomic response (e.g. raised heart rate), and a hypothalamic-pituitary-adrenal (HPA) -axis mediated response (e.g. cortisol release)\textsuperscript{193} (Fig. 11).

\textbf{Fig. 11.} The hypothalamic-pituitary-adrenal (HPA)–axis. CRH = corticotropin-releasing hormone, ACTH = adrenocorticotropic hormone, NE = norepinephrine, ACh = acetylcholine.
The stress response is generated through a network of brain structures, mainly the paraventricular nucleus of the hypothalamus, amygdale, and periaqueductal gray. These structures receive input from cortical structures, and visceral and somatic afferents.

2.5.2. Stress and intestinal disease

The influence of stress on intestinal disorders has for long been described by patients, but the actual effects of chronic stress on gastrointestinal diseases are still a matter of debate. However, studies are providing increasing evidence that chronic stress plays an important role in intestinal physiology, including more importantly mucosal barrier dysfunction.

There are several studies present confirming the importance of stress in gastrointestinal disease in humans. Barclay and Turnberg showed in the late 1980’s that psychological stress, induced by dichotomous listening or cold-induced hand pain, reduced mean net water absorption and transformed net sodium and chloride absorption to secretion. These effects were inhibited by atropine, suggesting the involvement of cholinergic neurons (achetylcholine). Ten years later, Santos et al extended this model and found that jejunal water secretion induced by cold pain stress was associated with luminal release of histamine and tryptase, typical mast cell mediators. These findings pointed to an interaction between the intestinal mucosa, mast cells, and central nervous system during stress in humans.

Since then, numerous studies have been performed in this field, and it is now known that chronic stress can modulate the inflammatory activity in IBD. Severe stress episodes are an important risk factor for the development and reactivation of intestinal inflammation. Moreover, a significant association has been found between acute daily stress and
bowel symptoms (e.g. pain, nausea, and diarrhea) in patients with Crohn’s disease \textsuperscript{201}.

2.5.3. Animal stress models
Since it is hard to study the effects of psychological stress on mucosal function in humans, several animal models have been developed. The stress model can be applied as acute or chronic stress. In acute stress, animals are exposed to stress at one session, and in chronic stress, exposure is repeated daily. The models include animals (most often rodents) which are exposed to restraint stress, water avoidance stress (WAS), swimming stress, crowding stress, social defeat stress or maternal deprivation stress \textsuperscript{195}. All these stress models have been developed with the intention to mimic psychological stress in daily life of humans. In WAS, animals are put on a small platform surrounded by water at room temperature for 20-60 min at one session (acute) or repeated daily (chronic) (Fig. 12).

\textbf{Fig. 12.} Rat submitted to water avoidance stress.
**Acute stress**

Acute stress in rats has shown that stress-induced mucosal dysfunction mainly involves corticotropin-releasing hormone (CRH), mast cells and cholinergic neurons\(^\text{202-204}\), and that stress increases the intestinal permeability to macromolecules without causing changes in gut morphology\(^\text{205-207}\). In an early study by Saunders et al\(^\text{205}\), rats submitted to acute restraint stress showed increased conductance and enhanced jejunal permeability to the paracellular markers mannitol and \(^{51}\text{Cr-EDTA}\). In subsequent experiments the stress-susceptible strain Wistar-Kyoto rats (rats with low cholinesterase activity) revealed an enhanced jejunal permeability after stress compared to the parent Wistar strain\(^\text{203}\). The fact that atropine inhibited the stress response further confirms the involvement of cholinergic neurons in stress. These experiments were followed by studies on macromolecular uptake by Kiliaan et al\(^\text{206}\) that could show an increased uptake of HRP in jejunum after stress. Stress-induced increases in colonic permeability have been reported as well\(^\text{207,208}\). These studies suggested a role for CRH in colonic epithelial pathophysiology and in another study by Pfeiffer et al\(^\text{209}\) increased paracellular permeability was seen after cold restraint stress and seemed to be induced by cholinergic neurons and mast cells.

**Chronic stress**

Several studies have shown that repetitive stress give more pronounced gut changes than acute stress. Rats submitted to 5-10 days of 1 hour WAS reduced their food intake and lost weight\(^\text{210}\). Further chronic stress increased the Isc, conductance and macromolecular permeability and also gave raise to bacterial attachment, mast cell hyperplasia, mitochondrial swelling and initiation of inflammation\(^\text{211,212}\). Mast cell-deficient rats exposed to chronic stress also lost weight but showed no signs of changes.
in epithelial function and gut morphology \cite{211}, confirming an important role for the mast cells in barrier function.

**The involvement of mast cells and neuropeptides in stress**

Several studies have confirmed the role of mast cells in stress-related changes of intestinal mucosal function. Castagliuolo et al \cite{213} found increased levels of the mast cell protease RMCPII during acute restraint stress in rats and the stress-induced increase in mucin was inhibited by adding mast cell stabilisers \cite{207}. Mast cells are often found close to neurons and are activated by neuropeptides, such as CRH, substance P and acetylcholine \cite{214}. CRH is known to be involved in the increased intestinal permeability seen after stress \cite{211} and peripheral injection of CRH mimics stress-induced permeability changes in rats \cite{207,215,216}. There are very few studies present regarding the role of vasoactive intestinal peptide (VIP) in mucosal stress and barrier function. However, recent studies showed increased VIP levels in mouse ileum after acute psychological stress \cite{217}, and in a human cell-culture model, a neural activated increase in paracellular permeability was inhibited by blocking of the VIP receptor \cite{218}.
3. AIMS OF THE THESIS

As mentioned in the introduction, the earliest observable signs of Crohn’s disease are aphtoid ulcers originating over the FAE. Crohn’s disease is associated with an increased immune response to bacteria, and the disease course can be altered by stress. With this in mind, together with the knowledge that FAE is an entry site for antigens and bacteria, we set up an overall aim to elucidate the effects of stress on the FAE and to reveal the role of FAE in the development of Crohn’s disease.

The specific aims of the separate studies were to:

I. Elucidate if the FAE barrier function could be modulated by stress. For this we set up an animal stress model and investigated the functional and morphological effects of stress in rat FAE.

II. Establish a technique for the identification of FAE in human ileal tissue and characterise the normal barrier properties of human FAE and compare it with regular VE.

III. Investigate the FAE barrier in patients with Crohn’s disease, using non inflammatory and inflammatory controls, respectively.

IV. Elucidate the mechanisms involved in the stress-induced increase in FAE permeability and to map the chemical coding of neuropeptides in rat FAE and Peyer’s patches.
4. **Subjects and Methodology**

For information on the statistics used, and other details on subjects and methodology, see the individual papers.

4.1. **Animals**

Since we have prior experience from stress experiments in male Wistar rats, we chose to work with this strain in the animal studies (Paper I and IV). From an immunological view, mice would have been preferable, however, from previous research we know that stress experiments in mice are difficult to perform.

A total of 102 rats, weighing 150-200 g at arrival, were acclimatised for one week and then handled daily for two weeks before the experiments started. The animals were kept in pathogen free conditions with a 12:12 h light:dark cycle and fed ad libitum on a diet of standard rat pellets and tap water. Studies were approved by the Animal Ethics Committee of the Faculty of Health Sciences at Linköping University, Sweden.

4.2. **Patients**

The study group in Paper II consisted of 19 patients undergoing surgery for right-sided colon cancer and 11 healthy persons undergoing colonoscopy for the surveillance of colonic polyps.

The study population in Paper III included 14 of the patients undergoing surgery for colonic cancer in Paper II, and 10 of the healthy persons undergoing colonoscopy. In addition, the study group consisted of 25 patients with Crohn’s disease (19 surgery and 6 follow-up colonoscopy) and 6 patients with extensive ulcerative colitis (2 surgery and 4 follow-up colonoscopy). All specimens were taken from the terminal ileum next to the ileocaecal valve or from the neo-terminal ileum, during surgery or
colonoscopy at the University Hospital of Linköping from March 2003 to May 2006. Both studies were approved by the Committee of Human Ethics, Linköping, University Hospital, and all subjects had given their informed consent. The colon cancer patients had no signs of generalised disease and none had received preoperative chemo- or radiotherapy. The Crohn’s disease patients had mild to moderate ileal inflammation and the ulcerative colitis patients showed signs of mild to severe inflammation in the colon.

The mean age for the Crohn’s disease patients was 48 (21-73) years, ulcerative colitis 61 (42-73), healthy controls 59 (38-75) and colon cancer controls 74 (47-85). Since intestinal permeability does not seem to be affected by aging \textsuperscript{116,219,220}, the age differences between the study groups are probably not important for the results.

4.3. Stress protocol

As mentioned in the introduction, there are several ways to generate stress in animals. In Paper I and IV, we used WAS, since it induces minimal physical stress and is considered a good psychological stress model of mimicking daily life stress in humans \textsuperscript{195}. In addition to studies on intestinal function, it has also been widely used in psychiatric research as a model of depression. Moreover, this model has previously been used in studies to achieve pure psychological stress effects on intestinal permeability \textsuperscript{211}. Prior to experimental start, rats were handled daily by the researcher for two weeks to decrease the influence of stress due to human contact, as we wanted the stress to be induced only by water avoidance, and evade stress effects in the control groups.

Rats were submitted to acute and/or chronic WAS. Rats to be stressed were placed on a platform (h=8 cm, d=6 cm) in a plastic container (h=56 cm, d=50 cm) with 25°C water, 1 cm below the platform. Control rats were either placed on a similar platform in a waterless container (Paper I)
or left in their cages (Paper IV). After one hour, the number of faecal pellets were counted as an indirect index of changes of colonic propulsive activity \(^{221}\). In chronic stress, this procedure was repeated for ten consecutive days, one hour per day. To minimise the effect of circadian rhythm, the procedures were started between 9 and 10 a.m. Before each session, rats were weighed.

Rats were anaesthesised by isofluran inhalation and 15-cm segments from the distal ileum (starting 5 cm proximal to the ileocecal valve) were taken out and immediately transported to the laboratory in ice-cold oxygenated Krebs buffer (115 mM NaCl, 1.25 mM CaCl₂, 1.2 mM MgCl₂, 2 mM KH₂PO₄ and 25 mM NaHCO₃, pH 7.35).

Blood for corticosterone analysis (Paper I) was collected by cardiac puncture, centrifuged, and stored at –70°C until further use for corticosterone assay (IDS, Boldon, U.K).

In Paper IV, animals were intraperitoneally injected with neuropeptide receptor antagonists or mast cell blocker, before being submitted to acute WAS. We chose to inject rats with anti-neurokinin-receptor 1 (NK-1R) and anti-CRH receptor (CRH-R), since the neuropeptides substance P and CRH have been shown to be involved in stress and permeability changes in VE. We also planned to study the effects of blocking the VIP receptor (VIPR), however, at the time of the study no such one was available.

### 4.4. Permeability studies

#### 4.4.1. Tissue preparation

In Paper I and IV, rat distal ileal specimens were, while immersed in Krebs buffer, stripped of external muscle and myenteric plexus, and FAE and VE were identified macroscopically.
In Paper II and III, human tissue from surgery and colonoscopy was used. Surgical specimens of distal ileum were immediately, after division of the ileocolic artery, put in Krebs, macroscopically reviewed by a pathologist, and transported to the laboratory. While rinsed in Krebs, specimens were stripped of external muscle and myenteric plexus. Tissue was then put with the mucosa up in a Petri dish and carefully stretched out with needles (Fig. 13). For identification of FAE, we modified a technique previously described for post-mortem ileal tissue 12. The dish was placed on a horizontal X-ray view box and by transillumination from below, regions of VE and FAE could be identified in a dissection microscope (Fig. 13).

**Fig. 13.** Identification of villus epithelium (VE) and follicle-associated epithelium (FAE) in surgical tissue. The border between VE and FAE is indicated by a stitched line. Note the regular pattern of villi (dark dots) in close apposition in the VE, compared to the more irregular pattern of multiple follicles surrounded by sparse villi in FAE.

Biopsies, identified as VE or FAE with magnification endoscopy, were taken at colonoscopy with a biopsy forceps without a central lance. They were directly put in a dish with Krebs, and epithelial type was identified with dissection microscopy as for surgical tissue.
Following the Ussing experiments, histological assessment verified epithelial type in each chamber (Fig. 14).

*Fig. 14. Histological assessment of VE (left panel) and an ileal lymphoid follicle (LF) and the overlaying FAE, following Ussing chamber experiments.*

Our intention in Paper I was to histologically identify M cells within the FAE of stressed rats and controls. There is one paper present that describes the identification of rat M cells by cytokeratine-8, in combination with the negative markers alkaline phosphatase and alcian blue. However, earlier studies have shown conflicting results and we were not able to obtain a specific staining with these markers. To date there is no reliable human M cell marker, and consequently, we did not stain for M cells in the FAE specimens of Paper II and III. However, a few markers have been suggested. One is Sialyl Lewis A Antigen, but we (unpublished observations) and others, have not been able to confirm this finding. Other suggested M cell markers are Cathepsin E and \( \beta-1 \) integrin. In a recent paper, we could show
that β-1 integrin, and also CD9, were several times higher expressed in FAE compared to VE, however, if the expressions were associated exclusively with the M cells and not to the other cells of the FAE could not be determined.

4.4.2. Ussing chamber experiments
Sections of VE and FAE were cut in appropriate sizes and mounted in modified Ussing chambers (Harvard apparatus Inc., Holliston, MA, USA) 227. When mounting FAE, the segments were carefully adjusted so that the patches covered the entire exposed tissue surface area of 9.6 mm² for rat tissue, 4.9 mm² for surgical specimens and 1.8 mm² for biopsies. Mucosal compartments were filled with 1.5 ml cold 10 mM mannitol in Krebs buffer and the serosal compartments were filled with 10 mM glucose in Krebs buffer. The chambers were kept at 37°C and continuously oxygenated, 95 % O₂ / 5 % CO₂ and circulated by gas flow. Before the experiments were started, tissues were equilibrated for 20-40 min in the chambers to achieve steady state conditions in PD, with a replacement of 37°C mannitol or glucose buffer at 20 min. The Isc, TER and PD were monitored using one pair of Ag/AgCl-electrodes (Ref 201, Radiometer, Copenhagen, Denmark) with 3M NaCl / 2 % agar-salt bridges and one pair of current-giving platinum electrodes. The chamber experiments were performed in open circuit conditions, and a four electrode system was used as previously described 228. The electrodes were coupled to an external six channel electronic unit with a voltage controlled current source. PD, Isc and TER were obtained as described by Karlsson et al 229. Data sampling was computer controlled via an A/D D/A board (Lab NB, National Instruments, USA) by a program developed in Lab View (National Instruments). Every second minute, direct pulses of 1.5, -1.5, 3, -3 and 0 µA with duration of 235 ms were sent across the mucosal specimens and the voltage response was measured. In each
measurement the mean voltage response of eight recordings was calculated. By this procedure the influence of AC disturbances of 25-100 Hz were eliminated. A linear least-squares fit was performed of the current (I)-voltage (U) pair relationship: \( U = PD + \text{TER} \times I \). From the slope of the line, TER was obtained, and PD from the intersection of the voltage. Tissue conductance was then calculated by inverting the TER values. All parameters were calculated during the 30-90 min period. Monitoring of FAE tissues revealed a higher TER compared to VE specimens. This probably refers to a thicker tissue in FAE segments due to the Peyer’s patches. Further, we observed a lower TER in biopsies compared to surgical specimens which probably is due to a thinner subepithelial tissue layer in biopsies. Conventional TER measurements, as used in Paper I-IV, give good assessments of changes over time, but miscalculations of TER, and also Isc, can be made when comparing different patients or epithelial types. Techniques that separate epithelial from subepithelial resistance are needed to fully elucidate differences in TER between different tissues.

4.4.3. Permeability markers

**HRP and \(^{51}\text{Cr-EDTA}\)**

In all Papers, we chose to use the 45 kD protein antigen HRP as a marker of protein uptake. HRP is known to, under normal circumstances, be taken up through the cells via macropinocytosis \(^{158,159}\), and has the antigenic potential to initiate immune responses in humans. HRP is easy to detect and has previously been used for permeability studies in Ussing chambers. One more advantage of HRP is its possibility to be detected by electron microscopy.

As a paracellular marker we chose to use the inert probe \(^{51}\text{Cr-EDTA}\). The EDTA molecule is known to pass between the cells via the paracellular
The binding of EDTA to the radioactivity labelled Cr is very strong, which assures that the Cr passage is equal to the passage of EDTA, and no Ca\(^{2+}\) can bind to EDTA to give detergent effects. Similar to HRP, \(^{51}\text{Cr-EDTA}\) is a widely used permeability marker in Ussing chambers.

HRP and \(^{51}\text{Cr-EDTA}\) were added to the mucosal side to a final concentration of 10\(^{-5}\) M and 34 \(\mu\text{Ci/ml}\), respectively. Serosal samples (300 \(\mu\text{l}\)) were collected at 0, 30, 60 and 90 min after start. An aliquot from each sample was saved for HRP analysis and the remainder was placed in a gamma-counter for \(^{51}\text{Cr-EDTA}\) measurements. Permeability was calculated during the 30-90 min period for both markers. \(^{51}\text{Cr-EDTA}\) permeability was given as \(P_{\text{app}}\) (apparent permeability coefficient; \(\text{cm/s} \times 10^{-6}\)), and HRP permeability presented as transmucosal flux (pmol/h/cm\(^2\)).

**E. coli K-12 and HB101**

Since we wanted to study bacteria that under normal circumstances do not invade the mucosa, we decided to use bacteria from non-pathogenic *E. coli* strains. Furthermore, increased numbers of *E. coli* has been found in Crohn’s disease mucosa. At Molecular Probes, The Netherlands, chemically-killed FITC-conjugated *E. coli* K-12 BioParticles are available. These bacteria are killed with paraformaldehyde in a way that stops their reproduction but retains antigenicity. They have previously been used for phagocytosis studies \(^{230}\) and should be a good model of bacterial uptake. Chemically-killed *E. coli* K-12 were used in all papers.

In addition, in Paper II and III, we used live green fluorescent protein-incorporated *E. coli* HB101 (One Shot® TOP10 Competent Cells, Invitrogen, CA, USA). For details on the incorporation, see Paper II.

Bacteria were added to the mucosal side to a final concentration corresponding to 1.0x10\(^8\) CFU/ml, i.e. the normal concentration of bacteria in the ileal lumen. After 90 and 120 min, serosal compartments
were collected and analysed at 488 nm in a fluorimeter (Cary Eclipse, Varian) where 1 unit refers to 1.5x10^6 CFU/ml.

**Doxantrazole, CRH and VIP**

In Paper IV, tissues were exposed to neuropeptides and mast cell stabiliser in the serosal chamber. We chose to study the effects of CRH and VIP peptides, since their involvement in stress and permeability changes in VE have been described previously. In the chambers 10 μM of the mast cell stabiliser doxantrazole (Sigma-Aldrich) was added. After 20 min, HRP and ^51^Cr-EDTA, or *E. coli* K-12 was added to the mucosal side while in the serosal chambers 1 μM CRH (Sigma C-3042), or 0.1 μM VIP (Sigma V-3628) were added. Serosal samples were collected at 0, 30, 60, 90 and 120 min, with replacement of Krebs buffer containing the blockers at starting concentrations.

4.5. **In vitro co-culture model of FAE**

In Paper II we used a modification of the co-culture model of FAE originally described by Kerneis et al. The model involves co-culture of Peyer’s patch lymphocytes and intestinal epithelial cells, that is thought to trigger the conversion of the epithelial cells to M cells. We obtained the model FAE by adding 5x10^5 Raji B cells (ATCC, ML, U.S.A) to the basolateral chamber of 14-day-old Caco-2 cell monolayers. The co-culture was then maintained for 4-5 days before onset of experiments.

To investigate the mechanisms behind the increased HRP uptake in FAE, we decided to inhibit the transcellular pathway. For this matter, we used the amiloride analogue 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), which inhibits the sodium-hydrogen exchange at the apical membrane, thereby reducing the uptake. Previous studies have shown that EIPA inhibits transcellular HRP uptake via pinocytosis, but not via clathrin-coated pits.
or via receptor-mediated uptake. In the experiments of Paper II, HRP, with or without EIPA, was added to the apical side of the cells, samples were withdrawn at regular time intervals, and stored at -20°C until analysed by confocal microscopy.

To study the mechanisms involved in the increased bacterial uptake in FAE we decided to inhibit the microtubule and actin filaments. For this purpose we used cytochalasin D, which is an F-actin polymerization inhibitor, and colchicine, which is known to block the microtubule transport. E. coli K-12 were added to the apical side of the cells, with or without inhibitors. After 20 min, the cell monolayers were washed, fixed in 4 % formaldehyde, washed again and stored for visualisation via confocal microscopy.

### 4.6. Immunohistochemistry

In Paper IV, immunohistochemistry was used to identify the neuropeptides VIP and CRH, and their receptors in rat FAE. Control sections were obtained from consecutive sections present on the same slide as the samples, which ensured a negative control for background and unspecific binding. The controls were added secondary, but not primary antibody. To confirm the nerve-mast cell interaction observed in the physiological experiments, mast cells were co-stained with May-Grünwald/Giemsa, which stains the nucleus and granule of the mast cells, respectively.

During antibody optimisation it was revealed that the rat tissue had very high levels of endogenous peroxidase, disturbing the actual peptide staining. However, when blocking tissues with 10 % hydrogen peroxidase in methanol, the endogenous peroxidase activity could be diminished. This rather tough treatment affected the histology resulting in partly damaged tissue with epithelium separated from underlying...
tissue. The immunohistochemical procedure was performed according to Fig. 15.

**Fig. 15.** The immunohistochemical procedure used to identify expressions of neuropeptides and their receptors in rat tissue.

To augment the antigen expressions in the tissue, we used a primary antibody followed by a biotinylated secondary antibody. Further, we utilised the ABC technique, where complexes that bind to the biotin molecule are built up to enhance the antigen expressions. The expressions were visualised by DAB staining.

### 4.7. Microscopy

**Light microscopy**

In Paper I, rat tissues were stained with May-Grünvald / Giemsa in order to count the number of mast cells. Cells identified as mast cells were counted in three stressed rats and three controls, three tissues per rat. Slides were coded, which ensured an objective evaluation.
In Paper IV, light microscopy was used to identify neuropeptides and their receptors in normal rat tissue. Co-staining for mast cells was done with May-Grünvald / Giemsa.

Confocal laser scanning microscopy
In Paper I-III, confocal microscopy was used to study bacterial uptake into rat and human tissue. Confocal microscopy is a variant of light microscopy with many advantages compared to the regular light microscope. For example, in a confocal microscope the illumination and detection is focused on the same point of the tissue, resulting in improved resolution and reduced degree of out-of-focus information. Thereby information can be collected from very thin specimens.
Tissues in Paper I-III were prepared for confocal microscopy as described in the respective paper.

Transmission electron microscopy
In Paper I-III, electron microscopy was used to study the passage routes for HRP and *E. coli* in rat and human tissue. The electron microscope uses a focused beam of electrons instead of light to create an image of the specimen. As electrons have much shorter wavelengths than light, it is possible to achieve magnification several thousands times higher than in a light microscope. When electrons pass through the specimen, interactions of heavy atoms will cause electrons to loose energy and deviate from its original direction. These interactions are then detected and transformed into an image.
Tissues for HRP studies were fixed in 2 % glutaraldehyde, incubated with DAB, postfixed in 1 % osmium tetroxide, embedded in Epon plastic, sectioned and stained with lead citrate and uranyl acetate. Tissues for bacterial passage studies were processed in the same way as for HRP, except for incubation with DAB.
5. RESULTS

Detailed descriptions of the results are given in the respective paper.

Paper I

Rats were submitted to acute or chronic stress to study the effects of stress on the FAE, and thereby elucidate if the FAE barrier function could be modulated by stress. Since this was the first study of FAE, we optimised the Ussing chamber technique for studies of FAE and Peyer’s patches before experiments started. Our results showed that FAE is responsive to both acute and chronic stress, illustrated by changes in electrophysiology and permeability. Acute stress increased the Isc, conductance, and HRP passage. Chronic stress enhanced the Isc and passage to HRP and E. coli. In addition, results revealed a significantly augmented permeability in FAE compared to VE. After chronic stress the HRP passage in FAE was over three times higher than in VE (Fig. 16).

![Graph]

**Fig. 16.** Rats were submitted to stress (filled bars) or non stress (open bars). Segments of villus epithelium (VE) and follicle-associated epithelium (FAE) were mounted in Ussing chambers and horseradish peroxidase (HRP) passage was measured over time. Results are given as the 30-90 min passage.
The enhanced uptake in FAE was further emphasised by a significantly enhanced *E. coli* passage compared to VE, with a passage six times higher in FAE than in VE after stress. Moreover, after 120 min exposure in Ussing chambers it was increased more than 30-fold in FAE of stressed rats compared to unstressed (Table 1). The bacterial uptake was confirmed by confocal microscopy.

**Table 1.** Effects of chronic stress on bacterial passage. Rats were submitted to stress or non stress (controls). Segments of villus epithelium (VE) and follicle-associated epithelium (FAE) were mounted in Ussing chambers. Passage of chemically-killed *E. coli* K-12 was studied over time. *P < 0.005 vs. controls FAE, †P < 0.05 vs. stress VE, ‡P < 0.05 vs. controls VE.

<table>
<thead>
<tr>
<th>Group, tissue</th>
<th>120 min passage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress, FAE</td>
<td><strong>47.9 (17.6 ± 94.7)</strong>&lt;sup&gt;a&lt;br&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control, FAE</td>
<td>1.4 (0.9 ± 5.1)</td>
</tr>
<tr>
<td>Stress, VE</td>
<td>7.8 (6.4 ± 19.3)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control, VE</td>
<td>5.0 (3.0 ± 5.8)</td>
</tr>
</tbody>
</table>

Electron microscopy revealed ultrastructural changes in FAE induced by chronic stress. This was indicated by areas of abnormal epithelial cell morphology with vacuoles. Within the FAE, M cells with internalised HRP in direct contact with lymphocytes could be identified (Fig 17 a, b).
**Fig. 17 a, b.** M cells (M) between enterocytes (E) in the follicle-associated epithelium of rat ileum. L = lymphocyte. Arrows indicate internalised horseradish peroxidase molecules.

*In conclusion,* we found that chronic stress modulated the barrier function of FAE allowing uptake of normally non-invasive luminal microorganisms. An enhanced bacterial uptake may lead to an increased antigen load and presentation in the dome area of the Peyer’s patches. Thereby a local inflammatory reaction could be initiated, eventually leading to intestinal inflammation. This may have implications for stress-related events in IBD.

**Paper II**

In this paper we wanted to establish a model for studies of human FAE and Peyer’s patches.

Initially we developed a technique for the identification of FAE in ileal surgical specimens from patients with colonic cancer by transilluminating the stripped mucosa from below in a dissection microscope. We were also able to identify FAE in biopsies, using similar techniques. Further, we could show that surgical specimens and biopsies
can be considered equal regarding permeability, as Ussing experiments showed similar permeability and electrophysiology in both techniques. Passage of HRP was higher in FAE than in VE (Fig. 18). The same pattern was seen in transmucosal passage of *E. coli* K-12 and *E. coli* HB101 (Fig. 19). The permeability to $^{51}$Cr-EDTA was equal in VE and FAE.

**Fig. 18.** Horseradish peroxidase (HRP) passage in villus epithelium (VE) and follicle-associated epithelium (FAE) of human ileum.

**Fig. 19.** *E. coli* K-12 and HB101 passage in human villus epithelium (VE) and follicle-associated epithelium (FAE). Median (25-75th interquartile range).
In vitro co-culture experiments and electron microscopy revealed actin-dependent, mainly transcellular, uptake of E. coli K-12 into FAE. Furthermore, the increased HRP passage in FAE was inhibited by blocking macropinocytosis.

In conclusion, we found an enhanced antigen and bacterial uptake, but similar paracellular permeability, suggesting functional variations mainly of transcellular transport in the FAE. Our results show that human ileal FAE is functionally distinct from regular VE, making it more prone to interaction with, and transport of, antigens and bacteria.

Paper III
This paper was designed to characterise the FAE barrier function in patients with Crohn’s disease. We used patients with colonic cancer or healthy volunteers as non-inflammatory controls, and patients with ulcerative colitis as inflammatory disease controls.

Initial morphological studies of paraffin-embedded tissues showed that the FAE of Crohn’s disease was prone to interact with bacteria. Fluorimetric measurements showed increased transmucosal passage in Crohn’s disease of both non-pathogenic E. coli strains. The passage of K-12 was almost doubled in Crohn’s disease (4.9 [4.2-5.3] units) compared to both ulcerative colitis (2.7 [2.1-3.8]) and controls (2.8 [2.4-3.4]), P < 0.002. The HB101 passage was also significantly increased in Crohn’s disease (6.7 [6.5-11.8]) compared to both ulcerative colitis (3.5 [2.9-5.3]) and controls (4.6 [4.0-5.3]), P < 0.002. In VE, there was no difference in flux between the patient groups for any of the strains.

Bacterial exposure induced changes in FAE conductance, suggesting that the E. coli affected barrier function of Crohn’s disease mucosa. Following bacterial uptake, there was an increased percentage of E. coli co-localising with dendritic cells in the mucosa of Crohn’s disease (45 [20-67] %)
compared to controls (14 [0-33] %), $P < 0.0001$, and augmented tissue release of TNF-α.

In conclusion, patients with Crohn’s disease, but not those with ulcerative colitis, exhibited an increased transmucosal uptake of non-pathogenic *E. coli* across the FAE, compared to non-inflammatory controls. Moreover, we found a higher amount of dendritic cells in Crohn’s disease and enhanced release of TNF-α. Our findings may have implications for the loss of tolerance to the normal gut flora in Crohn’s disease patients.

**Paper IV**

Rats were submitted to acute stress to elucidate the mechanisms behind the stress-induced increase in FAE permeability shown in Paper I. Prior to stress, rats were intraperitoneally injected with blockers for substance P (anti-NK-1R), CRH (anti-CRH-R) or mast cells (doxantrazole).

Results showed inhibition of stress effects in FAE when blocking the mast cells and receptors for CRH prior to stress. In FAE, the stress-induced increases in permeability to HRP (56.0 ± 14.4 pmol/h/cm² vs. controls 6.6 ± 1.9) and *E. coli* K-12 (23.3 ± 2.8 units vs. controls 4.3 ± 0.7) were diminished by anti-CRH-R and doxantrazole, however, the anti-NK-1R had no significant effect.

In VE, the increased *E. coli* K-12 passage after stress (7.1 ± 1.6 vs. controls 2.1 ± 0.4) was diminished by doxantrazole and anti-CRH-R, whereas there was no effect of anti-NK-1R. The increased HRP passage after stress (18.9 ± 6.9 vs. controls 6.6 ± 3.6) was only blocked by doxantrazole. Our results further showed that stress effects on permeability were mimicked by exposing both VE and FAE tissues to CRH and VIP in Ussing chambers. When also pretreating with doxantrazole, these effects were inhibited.
The physiological results were confirmed by the findings of CRH and VIP, and their receptors, within the Peyer’s patches, SED, and adjacent villi. Expressions of VIPR1 and CRH-R1 were found on mast cells located in the adjacent villi and surrounding the follicles, and more seldom inside the follicles and close to the FAE (Fig. 20 a, b)

**Fig. 20.** Mast cells with vasoactive intestinal peptide receptor 1 (VIPR1) and corticotropin-releasing hormone-receptor1 (CRH-R1) expression, respectively, in rat Peyer’s patches. (a) VIPR1 expression on mast cells (arrows, 1-2) at follicle margins. Arrowheads indicate mast cells negative for VIPR1, FAE = follicle-associated epithelium. (b) A mast cell with CRH-R1 staining inside the follicle (arrow).
VIPR2 and CRH-R2 were expressed to a lower extent. Staining for VIP and CRH peptide revealed immunoreactive mast cells mostly in the adjacent villi and at follicle margins.

*In conclusion*, we found that the stress-induced increase in FAE uptake of antigen and bacteria was prevented by blocking the mast cells and receptors for CRH. Furthermore, VIP mimicked stress-induced permeability changes, and the changes were abolished by stabilising the mast cells. Our results may be of importance for the pathogenesis in stress-related intestinal disorders, and give clues to new therapeutic approaches.
6. DISCUSSION

Several studies have shown an enhanced uptake across the FAE and Peyer’s patches in animals. In fact, FAE is considered as an important entry site for antigens as well as bacteria. The earliest observable lesions in the development of recurrent Crohn’s disease are initiated as microscopic erosions at the FAE. Crohn’s disease is further associated with an increased permeability and immune response to bacteria, and the disease course can be altered by environmental stress. Thus, we hypothesised that the barrier dysfunction seen in Crohn’s disease could be generated by alterations in the FAE.

Initially, we wanted to elucidate if the FAE barrier was sensitive to stress. When designing Paper I, no animal or human studies were present investigating the effects of stress on the FAE barrier. However, we knew from previous studies that acute and chronic stress in rats could modulate the VE barrier, as indicated by enhanced permeability, increased mucin production, bacterial attachment, mast cell hyperplasia, mitochondrial swelling and initiation of inflammation. With this in mind, we decided to submit rats to acute and chronic physiological stress to evaluate the effects on FAE. Our results revealed that the FAE barrier was sensitive to stress, shown by changes in electrophysiology and increased uptake of luminal antigens and bacteria. Hereby we could, for the first time, demonstrate an enhanced permeability in FAE after stress, and also higher permeability increase in FAE compared to VE.

In a follow-up study we wanted to confirm the increased FAE permeability in humans. Results showed a considerably more pronounced bacterial and antigen uptake also in human FAE, compared to VE. Since we could show that stress increased FAE permeability, and
stress is a contributing factor to IBD and Crohn’s disease, we speculated that there could be an altered FAE function in patients with Crohn’s disease. Our results showed that patients with Crohn’s disease displayed an enhanced bacterial passage in FAE compared to non-inflammatory and inflammatory controls.

In the present studies, we found an enhanced antigen and bacterial permeability in FAE compared to VE in both rats and humans. In addition, we found that stress further increased the permeability in FAE. The HRP passage was increased almost four times in FAE after stress compared to 1.8 times in VE, and bacterial uptake increased 30 times in FAE after stress, as compared to only 1.6 times in VE. This suggests that FAE is more vulnerable to stress than VE, and it might be speculated that a major part of the bacterial translocation that occurs in stress may be due to invasion across the FAE. When comparing the permeability in Crohn’s patients with that in non-inflammatory controls, a higher bacterial permeability in FAE was seen. However, the HRP passage was similar, suggesting different mechanisms of the FAE barrier dysfunction during stress and inflammation.

The higher passage in FAE compared to VE in the normal situation probably refers to the different surface characteristics, making FAE more accessible for luminal antigen and bacteria. In addition, the presence of M cells within the FAE contributes to the increased permeability. However, the fact that the FAE permeability increases after stress, and is enhanced in Crohn’s disease, indicates that something occurs in the epithelium that further facilitates for luminal antigens and bacteria to cross the barrier. Since a higher M cell number has been found during inflammation, the increased permeability in Crohn’s disease could, at least partly, be due to an increased M cell number within the FAE. One could further speculate that the numbers are also increased
after chronic stress, which could explain the enhanced permeability. Thus, it would have been highly interesting to verify the M cells number in that FAE tissue investigated in our studies, but as already mentioned, there is no reliable rat or human M cell marker, which hampers the possibility to identify the M cells present in our FAE tissue investigated. Contradictory to the hypothesis that the increased permeability is due to M cells in the FAE, is one paper showing an M cell independent mechanism. In a co-culture model of mice dendritic cells and human epithelial cells, it was shown that dendritic cells directly can sample luminal antigen by unzipping tight junctions and extending their dendrites through the intact epithelium. However, this observation has not yet been reproduced in human tissue.

The importance of FAE in permeability might be questioned due to its smaller surface area compared to VE. The VE contains numerous folded villi that increase the surface area considerably compared to FAE. However, the smaller surface area is highly compensated by the larger capacity of the FAE cells to sample luminal content, and the importance of the underlying Peyer’s patches for initiation of immune responses. When antigen and bacteria enter the FAE and M cells, they are transported across the cells, and delivered to antigen-presenting cells in the Peyer’s patches to induce inflammatory responses. Lymphocyte-containing M cell pockets decrease the travelling distance from the apical to the basolateral side, which further improves the passage in FAE. In VE, the major part of entering content is degraded on their way through the cells via for example lysosomal events, and only a minor amount reaches all the way through intact.

From our results it may be suggested that in Crohn’s disease, antigen and bacteria enter the FAE barrier at a higher rate due to a disturbed barrier
function. The question is whether the disturbed barrier is caused by a primary epithelial defect, or if it is the other way around and the increased permeability is a consequence of an early immune activation.

In the first theory, it could be speculated that patients with Crohn’s disease have a genetically driven disrupted barrier function leading to increased uptake of luminal antigens and bacteria, which are phagocytosed and presented to immune cells that starts to produce inflammatory mediators. After secretion, cytokines and chemokines attract other inflammatory cells that start to produce even more inflammatory mediators. Finally this could lead to further destruction of the epithelial cells, by the inflammatory mediators, affecting epithelial defence and control of permeability. This theory is strengthened by studies showing increased permeability in healthy relatives of patients with Crohn’s disease 18,20,21.

The second theory says that immune cell activation and inflammation precede the barrier disruption. The importance of immune cell activation in barrier dysfunction has been confirmed by several studies. For example TNF-α and IFN-γ have shown to increase both paracellular and transcellular permeability 170,237,238 and studies have shown that the barrier dysfunction in Crohn’s disease is TNF-α-dependent 170,239,240. Furthermore, it has been demonstrated that monocytes from patients with Crohn’s disease secrete TNF-α upon bacterial stimulation 140, and mast cells of Crohn’s disease mucosa are major producers of TNF-α 241,242. A study speaking in favour of inflammation coming first is that 54 % of healthy relatives to Crohn’s disease patients have subclinical inflammation, measured as levels of calprotectin 243. Moreover, in a rat model of intestinal inflammation it was shown that the rats developed inflammation without getting increased permeability 244.

Both of the theories make sense. It could be that in Crohn’s disease the epithelial barrier is initially to some extent disrupted, for example due to
an abnormal response to bacteria. This leads to increased passage, immune activation and subsequently elevated levels of for example TNF-α, IFN-γ and IL-4 \textsuperscript{142,170,237,238} that further destroy the barrier and increase the permeability.

The importance of the luminal microflora in Crohn’s disease is well documented \textsuperscript{41,42}. For example studies have shown increased number of adherent-invasive \textit{E. coli} in Crohn’s disease mucosa \textsuperscript{43,44}. The significance of bacteria in intestinal inflammation is further strengthened by the fact that rats susceptible for intestinal inflammation did not develop IBD when bred under germfree conditions \textsuperscript{49}.

The novel findings in Paper III, an increased uptake of non-pathogenic bacteria in FAE despite an equal permeability to \textsuperscript{51}Cr-EDTA and HRP, point to the importance of FAE in Crohn’s disease and highlight the importance of FAE-bacterial interactions leading to inflammation. Since the FAE tissues of patients with ulcerative colitis showed no increase in bacterial passage, the enhanced bacterial transport may be specific to the FAE of Crohn’s disease. What are the mechanisms behind this increase? Possible explanations could be the involvement of receptors, referred to as pattern-recognition receptors (PRR), that recognise the so called pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharide (LPS) and peptidoglycans, \textsuperscript{245} The two major groups of PRRs are the NOD-containing proteins and toll-like receptors (TLRs).

The best characterised NOD proteins relevant to the intestinal physiology are NOD1 and NOD2, where NOD2 recognises structures in a wider range than NOD1, and is more restricted to the small intestine. Although several potential signalling pathways involved in inflammation and innate immunity linked to NOD2 activation have been demonstrated, the physiologic functions are less well understood. Under normal conditions the NOD2 expression is low, however, during inflammation the
expression increases, which could suggest a role for altered microbial sensing in the increased bacterial uptake in Crohn’s disease. Mutations in the NOD2 gene lead to inhibited activation of the NOD2 protein, however, since none of the Crohn’s disease patients included in our study carried the mutation, one can presume that the NOD2 protein was unaffected.

TLR-4 is the best studied TLR and is essential for the recognition of LPS, and further limiting LPS responsiveness. A TLR-4 polymorphism and increased apical expression of TLR-4 has been found in both Crohn’s disease and ulcerative colitis. The overexpression of TLR-4 could result in LPS hyper-responsiveness leading to consecutive proinflammatory cytokine secretion which in turn stimulates TLR-4 expression and further inappropriate signalling in the presence of luminal LPS. It is known that TLR-4, is upregulated in FAE and M cells of mice, and may be involved in regulation of uptake of bacteria and microparticles. Therefore, the role of TLR-4 in the FAE dysfunction in Crohn’s disease should be elucidated in further studies.

Another way in which TLRs are involved in regulation of tolerance to commensal bacteria is through their effect on dendritic cells. After sampling of luminal content, dendritic cells migrate across the epithelium and by stimuli from bacteria via TLR signalling, maturation is induced. Dendritic cells are located in the SED for phagocytosis and presenting of antigen. It has been shown that they can squeeze in between epithelial cells through the tight junctions to sense and sample luminal microbes. Our findings in Paper III of increased bacterial uptake into dendritic cells in Crohn’s disease mucosa suggest that dendritic cells may play an active role in the immune cell-bacterial interaction leading to inflammation in Crohn’s disease.
Our results, demonstrating a disrupted FAE barrier function after stress in rats gave rise to Paper IV where we wanted to elucidate the role of mast cells and neuropeptides in the increased uptake. The significance of mast cells in stress-induced mucosal dysfunction of VE has been clearly demonstrated by several groups \(202,204,211,212\), and we could also confirm this in Paper I, where we observed a 3-fold increase in mast cell number in stressed rats compared to controls. Numerous studies have highlighted the role of CRH in stress \(207,215,216\), and some of them have demonstrated inhibited effects of CRH when blocking the mast cells, however, the importance of mast cells and CRH in stress-induced FAE permeability has not been reported. Our results showed inhibited stress effects when blocking the receptors for CRH, and mimicked stress-effects on permeability when exposing FAE to CRH peptide in Ussing chambers. In addition, when stabilising the mast cells with doxantrazole, the effects of both stress and CRH were inhibited, which points to that CRH is released during stress and increases permeability via activation of mast cells.

In addition, we could, in Paper IV, for the first time, identify VIP as a regulator of mucosal permeability. FAE exposure to VIP in Ussing chambers resulted in increased paracellular permeability and transcellular passage to antigen and bacteria. Furthermore, we found VIP peptide and VIP receptors both in and close to mast cells at follicle margins and in adjacent villi. The involvement of VIP in permeability has previously only been described briefly. In a co-culture model of human submucosa containing the submucosal neuronal network and human polarised colonic monolayers, the paracellular permeability was increased by electrical stimulation of submucosal neurons \(218\). The effects of the neuron activation were blocked by a VIP receptor antagonist, and reproduced by VIP peptide. This, together with our results regarding
VIP-induced increased permeability, suggest an important modulatory role for VIP in the regulation barrier function.

In addition to the importance of mast cells and neurons during stress, their roles in IBD have also been highlighted. Degranulation of mast cells, and increased numbers, have been found in mucosa from patients with ulcerative colitis 253 as well as Crohn’s disease 254. Moreover, elevated levels of histamine have been measured in gut lumen of Crohn’s disease patients. 255

There are only a few studies present regarding the consequences of neuronal changes on mucosal function in IBD. However, changes in neuropeptide innervation 256, and altered VIP 257;258 and substance P 259;260 expressions, have been found in mucosa from patients with ulcerative colitis and Crohn’s disease. There are no reports on immunoreactivity to CRH in Crohn’s disease, however, in mucosal inflammatory cells of ulcerative colitis, increased CRH expressions have been observed 261.

Even if our results demonstrate a role of VIP in FAE barrier function during stress, it can not be directly applied to the permeability changes seen in FAE of Crohn’s disease. However, several studies have shown a link between stress and IBD 191;194;199;200, and the fact that a disrupted barrier function can be induced in healthy rats, by submitting them to stress, suggests that stress may be implicated in the initiation, perpetuation, or exacerbation of the inflammation seen in IBD. Consequently, it could be speculated that VIP is important also in regulating the permeability in Crohn’s disease, not at least considering the increased VIP expression found in Crohn’s disease mucosa. However, further studies are needed to elucidate the role of VIP in FAE barrier function of Crohn’s disease.
In conclusion, this thesis presents novel insights into regulation of the FAE barrier, as well as into the pathophysiology of Crohn’s disease by demonstrating a previously unrecognised defect of the FAE barrier function in ileal Crohn’s disease. Further studies in a rat IBD model would be highly valuable to better understand the mechanisms involved, and the role of FAE in the interplay between stress and intestinal inflammation.
7. CONCLUSIONS

- The FAE barrier can be modulated by stress as illustrated by enhanced antigen and bacterial passage and ultrastructural changes after acute and chronic stress in rats.

- The more pronounced increase of transmucosal passage in FAE compared to VE after stress suggests that FAE is more stress-reactive and prone to interact with antigen and bacteria.

- Human FAE in biopsies and surgical specimens can be identified by transilluminating the mucosa from below in dissection microscope, and the techniques can be considered equal regarding permeability measurements in Ussing chambers.

- Human FAE exhibits a substantially higher antigen and bacterial passage compared to VE. In vitro experiments revealed an actin-independent passage of *E. coli* and HRP uptake via macropinocytosis.

- Patients with Crohn’s disease demonstrate a higher transmucosal uptake of *E. coli* K-12 and HB101 compared to non-IBD controls and patients with ulcerative colitis, suggesting that the FAE barrier is altered in Crohn’s disease by disease-specific mechanisms.

- Following bacterial uptake, patients with Crohn’s disease reveal a higher percentage of *E. coli* internalised by dendritic cells compared to non-IBD controls.
• The stress-induced increase in FAE permeability seen in rats is regulated by CRH and mast cells. Further stress effects on permeability can be mimicked in vitro by VIP, and the effects are abolished by blocking the mast cells.

• In rats, mast cells are present mainly in the adjacent villi, but also in follicles and SED. The neuropeptides CRH and VIP and their receptors are expressed in mast cells within these regions.
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9. **Svensk Sammanfattning**

Crohns sjukdom är en kronisk inflammatorisk tarmsjukdom av okänd orsak. Det tidigaste tecknet på Crohns sjukdom är mikroskopiska sår i det s.k. follikelassocierade epitelet (FAE) som täcker ansamlingar av immunceller i tarmen. FAE är specialiserat för att fånga innehåll från tarmen och transporterar det till underliggande immunnävnan. Denna funktion är viktig för att inducera skyddande immunsvar, men den utgör också en ingångsväg för sjukdomsalstrande bakterier. Crohns sjukdom är associerat med ett kraftigt ökat immunsvar mot bakterier, och sjukdomsförloppet kan ändras av stress.

Det övergripande syftet med avhandlingen var att studera effekterna av stress på FAE samt att undersöka rollen av FAE vid utvecklingen av tarminflammation, särskilt vid Crohns sjukdom.

Inledningsvis studerades effekterna av psykologisk stress på FAE. Stressade råttor uppvisade ökad genomsläpplighet av bakterier efter stress, och passagen var högre i FAE än i vanligt epitel. Efterföljande experiment visade att stressförändringarna i slemhinnan regleras via kortikotropinfrisättande hormon och mastceller. Vidare visade det sig att vasoaktiv intestinal peptid kunde efterlikna stressens effekter på genomsläppligheten, och att detta kunde förhindras genom att blockera mastcellerna.

Studier av tunntarmsslemhinna från patienter med icke-inflammatorisk tarmsjukdom och friska kontroller visade en högre passage av bakterier i FAE än i vanligt epitel. Hos patienter med Crohns sjukdom var bakteriepassagen genom FAE betydligt ökad jämfört med kontroller.

Resultaten från detta avhandlingsarbete visar att stress kan förändra upptaget av bakterier från tarmen via FAE, med mekanismer som innefattar kortikotropinfrisättande hormon och mastceller. Detta har getts nya kunskaper kring regleringen av slemhinnebarriären. Vidare presenterar denna avhandling nya insikter i sjukdomsuppkomsten vid Crohns sjukdom genom att påvisa en tidigare okänd defekt i barriärfunktionen i FAE.
10. References


patients enrolled in remission, Am.J.Gastroenterol., 95, 1213-1220 (2000).


[220] L. Blomquist, T. Bark, G. Hedenborg, and A. Norman, Evaluation of the lactulose/mannitol and 51Cr-ethylenediaminetetraacetic


[260] S. Mazumdar and K. M. Das, Immunocytochemical localization of vasoactive intestinal peptide and substance P in the colon from