Neuro-immune regulation of macromolecular permeability in the normal human colon and in ulcerative colitis

Conny Wallon

Division of Surgery
Department of Clinical and Experimental Medicine
Faculty of Health Sciences,
SE-581 85, Linköping, Sweden

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Cover:

Scanning electron microscopy of a mounted colonic biopsy in the Ussing chamber. Note the structure of the colonic crypt openings and the edge of the plastic slide.

SUPERVISOR
Johan Dabrosin Söderholm, Professor of Surgery,
Division of Surgery, Department of Clinical and Experimental Medicine,
Faculty of Health Sciences, SE-581 85, Linköping, Sweden

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“Medicine is a science of uncertainty and an art of probability.”

William Osler
1849-1919

Till min familj; Susanne, Felicia,
Sebastian och Christian
ABSTRACT

Background and aim: Persistent stress and life events affect the course of ulcerative colitis (UC) by largely unknown mechanisms. Regulation of epithelial permeability to antigens is crucial for the balance between inflammation and immuno-surveillance, and increased intestinal permeability has been shown in patients with ulcerative colitis. Corticotropin releasing hormone (CRH) has been implicated as an important mediator of stress-induced abnormalities in intestinal mucosal function in animal models. Further cholinergic signalling during stress has been reported to increase bowel ion secretion in humans and uptake of HRP in rodents via activation of mast cells.

The overall aim of this thesis was to examine the role of CRH-mediated and cholinergic signalling, and their interaction with mast cells and eosinophils, in the regulation of the mucosal barrier function in the normal human colon and in UC. In vivo studies or the use of surgical specimens for such studies have major shortcomings. Therefore a method with endoscopic biopsies in Ussing chambers was established for studies of protein antigen uptake and electrophysiology in human colonic biopsies, and used in subsequent investigations.

Materials and methods: In the four studies a total of 91 healthy volunteers, 3 patients with rectal cancer, and 15 UC patients were included. Biopsies from the sigmoid colon were assessed for macromolecular permeability (Horseradish peroxidase (HRP), and $^{51}$Cr-EDTA), and electrophysiology during challenge with sodium caprate (C10), CRH or carbachol. Experiments were repeated with CRH receptor antagonists, carbachol receptor antagonists, mast cell stabilizers and nerve conductance blockers in Ussing chambers. The biopsies were examined by electron and light microscopy for endocytosis of HRP, morphological changes and receptor expression. Moreover, the human mast cell line, HMC-1; was used in studying expression of CRH receptors on mast cells.

Results: Endoscopic biopsies of human colon were viable in Ussing chambers, and the technique was shown to be a reliable tool for studies of mucosal permeability to HRP. CRH stimulates transcellular uptake of HRP in human colon via CRH receptor subtypes R1 and R2 on subepithelial mast cells. Further, carbachol acts on muscarinic receptors, located on subepithelial eosinophils. Activated muscarinic M2 and M3 receptors on increased numbers of CRH-producing eosinophils in UC, lead to activation of mast cells and increased macromolecular uptake across the colonic mucosa. This signalling cascade is previously unrecognized, and may be involved in the inflammatory process in UC.

Conclusions: In conclusion, we have demonstrated a chain of events leading to increased permeability to the protein antigen HRP in biopsies from healthy volunteers and patients with UC. The important steps begin with a cholinergic signal to muscarinic receptors on the CRH containing eosinophils. The next step includes activation of CRH receptors on mast cells leading to degranulation and increased macromolecular uptake across the epithelium. This explanatory model will have implications for understanding of the pathogenesis of UC and future treatment of the disease.

List of papers

This thesis is based on the following papers;


LIST OF PAPERS

This thesis is based on the following papers;

I. Endoscopic biopsies in Ussing chambers evaluated for studies of macromolecular permeability in the human colon.

II. Corticotropin releasing hormone (CRH) regulates macromolecular permeability via mast cells in normal human colonic biopsies in vitro.

III: Carbachol regulates transcellular antigen permeability in human sigmoid colon biopsies in vitro.

IV. Cholinergic stimulation-induced release of CRH from eosinophils mediates increased macromolecular permeability in ulcerative colitis.
ABBREVIATIONS

Ach  Acetylcholine
Atr  Atropine
C10  Sodium caprate
Cch  Carbachol
CNS  Central nervous system
CRH  Corticotropin-releasing hormone
CRH-R1, R2  CRH receptor type 1, 2
ENS  Enteric nervous system
GI   Gastro-intestinal
Hex  Hexamethonium
HMC-1  Human mast cell line -1
HPA  Hypothalamic-pituitary-axis
HRP  Horseradish peroxidase
IBD  Inflammatory bowel disease
IBS  Irritable bowel syndrome
IL   Interleukin
Isc  Short circuit current
JAM  Junctional adhesion molecule
KRB  Krebs-Ringer bicarbonate buffer
M1, M2, M3  Muscarinic receptor type 1, 2, 3
MAGUK  Membrane associated guanylate kinase protein family
M-cell  Microfold or membranous cell
MLCK  Myosin light chain kinase
PD   Transepithelial potential difference
PKC  Protein kinase C
TER  Transepithelial electrical resistance
TJ   Tight junction
TLR  Toll like receptor
TNF-α  Tumor necrosis factor alpha
TTX  Tetrodotoxin
UC   Ulcerative colitis
VE   Vehicle
ZO-1  Zonula occludens protein type 1
4-DAMP  4-diphenylacetoxy-N-methylpiperidine methiodide
⁵¹Cr-EDTA  Chromium 51 ethylene diamine tetra-acetic acid
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1. INTRODUCTION TO ULCERATIVE COLITIS

History

Inflammatory diseases in the gastro-intestinal tract have been known for thousands of years. Descriptions of acute and chronic diarrhoea with or without blood goes back to the ancient world and is described in scripts between 640 BC and 170 AD by Hippocrates of Cos, Aretalus of Cappadocia and by Soranus of Ephesus.1 In the 16th century Thomas Sydenham described an intestinal disease as “the bloody flux”. The description resembles well with ulcerative colitis (UC). In The Medical Times and Gazette (1859), Wilks and Moxon described for the first time, in a case report, a patient with symptoms of a condition they named "Inflammation of the large intestine or idiopathic colitis".2;3 But it was not until 1888 the term ulcerative colitis was used for the first time when Hale-White distinguished different types of colitis in 60 patient cases.4 The diagnosis was based on patient history and stool analysis. In 1905 a new invention, the electrical sigmoidoscopy, could separate patients with and without visible recto-sigmoid inflammation.5 Today diagnosis of UC is based on well-established clinical, endoscopic and histological criteria.6 Recent advances in the detection of faecal and serologic markers and the use of wireless capsule endoscopy are promising diagnostic adjuncts for the near future.7

Epidemiology and symptoms

The peak incidence of UC occurs between the ages of 15 and 25 with a second peak incidence in the sixties. The incidence is 10-15/100000 per year (North America and Sweden) with a prevalence of 350/100000. The disease affects females more than males (1.2:1).5 There is also a geographic distribution of UC.9;10 UC occurs more commonly among Ashkenazi Jewish people than non-Jewish people. The major symptoms of UC are bloody diarrhoeas and abdominal pain. In mild disease stools are semi formed containing little blood and no systemic manifestations. In contrast, in severe disease, patients have frequent liquid stools containing blood, mucus and pus. Systemic manifestations include dehydration, anaemia, fever, weight loss and sometimes abdominal pain.

Aetiology

Genetics

Epidemiological studies show that ethnic background and family history of inflammatory bowel disease (IBD) are of importance in the susceptibility for UC. A positive family history is still the largest independent risk factor for the disease. In UC patients 5.7–15.5% have a first-degree relative with the same disease and 6.6% to 15.8% a first-degree relative with unspecific IBD.11 Furthermore, twin
studies show higher pair concordance rate in monozygotic twins with UC than dizygotic twins. A convincing evidence for genetic involvement in IBD is mutation on chromosome 6 (IBD3), which encodes the major histo-compatibility complex. Certain mutations in these loci might be associated with certain disease phenotypes or disease courses. The HLA haplotype DRB*0103 has, for example, been linked to a particularly aggressive course of UC and the need for surgery. Mutations on HLA-B27 or HLA-B35, and HLA-B44 or HLA-DRB*0103 have been linked to extra intestinal disease complications, such as arthropathy or uveitis.

**Immunology**

The balance of the normal colonic immune system is disturbed in UC. Under normal conditions the immune system acquires tolerance towards luminal antigens including commensal microflora. However, in IBD, luminal antigens gain access to the underlying mucosal tissue via a leaky barrier. The innate and adaptive subepithelial immune cells respond to the invading antigens and trigger an abnormal inflammatory response due to a false recognition of commensals as pathogens. This induces expression of different inflammatory activating receptors and stimulatory molecules. The toll-like receptors, specialized in recognition of microbes and their products, trigger the innate and adaptive antimicrobial response by intracellular signalling including transcription of Myd88 and induction of the inflammatory cascade. This is in line with a change in the immune cells functional status from tolerogenic to activation and promotes differentiation of naive T cells into effector T cells (i.e. Th1, Th17, and Th2) and natural killer T cells. In UC, naive T cells differentiate into aberrant Th2 (IL-5 positive) cells. IL-5 is also produced by mast cells. Natural killer T cells are probably the main source of IL-13 in UC. IL-13 is a key effector cytokine in UC, stimulating epithelial cell apoptosis, increase epithelial permeability due to changes in function of tight junction (TJ) via alterations of claudin-2 expression. The pro inflammatory cytokines secreted by activated effector T cells stimulate macrophages to secrete large amounts of tumour necrosis factor α (TNF-α), IL-1, and IL-6 which are known to increase epithelial permeability to antigens.

**Environment**

*Geographic and socioeconomic factors.* High incidence rates of UC are reported from the geographical northern hemisphere while low incidence rates are reported from South America, Africa and Australia. The highest incidences is shown in Scandinavia, The United Kingdom, The United States, and Canada. This indicates a gradient exists of the disease from north to south but it can also indicate variations in health care access and diagnosis confusion with other diarrhoeal diseases, more common in developing countries.

There are however indications that excessive sanitation might limit the exposure to environmental antigens of the mucosal immune system with impairment of immune maturation and tolerance which may lead to inappropriate immune responses to commensals and non pathogenic antigens later in life.
**Diet.** There is a weak association between food intake and IBD. Most dietary studies are of inferior quality due to poor patient compliance and interpretations of findings are difficult. Probiotics will be discussed under the treatment chapter.

**Smoking.** Several studies have shown beneficial effects of cigarette smoking in UC, with less frequent exacerbation of the disease. Experimental studies suggest increased mucus production with a thicker epithelial mucus layer, decreased production of nitric oxide and proinflammatory cytokines and improved colonic barrier function as an effect of nicotinic intake. However, in clinical studies, nicotinic patches and nicotinic enemas have failed to show efficacy in the management of UC.

**Psychological stress.** Long term ongoing psychological stress increases the risk of exacerbation in UC. Explanations include hyper-activation of the immune system and alterations in the colonic barrier. Psychological stress will be further discussed in the next chapter.

**Barrier function**

The intestinal epithelium is the first line of physical defence against invading micro-organisms. Therefore, increased antigen permeability across the mucosal epithelial barrier is a challenge for the innate immune system and could, in a dysfunctional immune system, trigger a vicious immune reaction leading to chronic intestinal inflammation. Increased intestinal permeability to antigens has gained increasing interest in IBD pathogenesis. Strong evidence for barrier dysfunction due to the downstream result of mutation in NOD2/CARD15 gene has been shown for Crohn’s disease but not for UC. In chronic and acute UC, increased mucosal macromolecular permeability due to disruption of the colonic barrier have been shown. Significant changes in the TJ structure with elevation of claudin 2 protein expression due to increased interleukin 13 production in UC patients may partly explain increased uptake of antigens. Studies have shown increased transcellular uptake of protein antigens in Crohn’s disease. Little is however known about transcellular uptake of antigenic proteins in UC. There are only a few studies describing increased transcellular uptake in UC. In the next chapter, the colonic intestinal barrier will be discussed in detail.

**Treatment**

**Surgery**

Surgery for UC was not possible until the end of the 19th century. Invention and improvement of anaesthetic techniques in general anaesthesia using ether made abdominal surgery possible. However, in the beginning of the surgical era, patients who underwent abdominal surgery were at great risk of infectious complications with high postoperative mortality. In 1893 AR Mayo-Robson established a colostomy in a woman with UC through which the colon could be irrigated. In the beginning of the 20th century appendicostomy or caecostomy, through which
irrigation was performed, were state of the art in the surgical treatment of UC. In 1913 J Brown and others were attracted to the idea of diverting the faecal stream from the colon by another invention, the ileostomy. However the stomas were made “flush” with the abdominal wall and no appliance to collect the faecal effluent was available. Further, the stomas were sutured to the skin with exposed ileal serosa leading to complications of stoma-strictures, infections and high output stomas. This occurred in more than 60 % of the patients operated on. The early ileostomy was regarded as a last resort operation and reserved for the critically ill patients, associated with a high mortality (> 30 %). There were also other publications of surgical techniques which appeared now and then in the beginning of the century. Lillienthal performed in 1903 an ileosigmoideoestomy in a young patient after five colonic segmental ressections and in 1912 Vignolo did a proctocolectomy with a segment of ileum sutured to the anal canal. However, these descriptions were few and not supported by the surgical society at that time. In 1944 the invention of the stoma bag by Strauss changed the predominate methods in surgical treatment of UC. It was now possible to collect the stoma effluents and the proctocolectomy with a stoma was increasingly popular. In 1951 and 1952 B Brooke published two important papers describing the surgical techniques in eversion of the ileostomy and single stage proctocolectomy with creation of a permanent end ileostomy. These papers had profound impact on the surgical society because the stoma construction solved the stricture, infectious and excessive fluid output complications. Patients now survived the acute relapse in UC but became ileostomates and whished for an alternative. Nils Kock at Sahlgrenska Sjukhuset in Sweden constructed a continent pouch ileostomy 1969. The internal reservoir is continent due to a valve mechanism and is emptied by intubation through a stoma and evacuated regularly. Another profound milestone in surgical treatment of UC began in 1947 with the paper by Ravitch and Sabiston who performed “anal ileostomies”. J Goligher improved the ileoanal anastomosis and reported the loop ileostomy to protect the anastomotic healing. However in 1978 Parks, Nicholls and Belliveau reported their experience with the ileal pouch anal anastomosis- IPAA, a s-shaped ileal internal reservoir. Today IPAA is the golden standard in curative surgery for UC, but with new potent anti-inflammatory treatments, the ileo-rectal anastomosis is increasing in popularity due to the lesser surgical trauma and reduced risk of infertility.

**Medical treatment**

*Cortico-steroids.* In the beginning of the 20th century different unproven regimes of various drugs were the only medical treatment provided for UC patients. However in 1954 S. Truelove and L. Witt published their work on the effects of cortisone in UC. This was the first clinical study in the history of gastroenterology and it revolutionized medical treatment of UC. Still today cortisone constitutes the first line of treatment in relapses of UC. A few years later Truelove el al showed the value of hydrocortisone locally in distal UC.
5-aminosalicylic acid (5-ASA). In the 1930’s in Sweden the first female professor of medicine N. Svartz developed a drug for rheumatoid arthritis (RA), sulfasalazine. Some of the RA patients were also affected by UC and after treatment with sulfasalazine, the intestinal symptoms were significantly improved.35 Today 5-ASA, the active substance of Sulfasalazine is the first line medication for maintenance of remission.

Immunosuppressants. In 1960 Calne et al described in a paper on renal transplantation mercaptopurine and later azathioprine as immunosuppressants that could be used in modulating the immune system.36 Two years later, Bean et al published a paper on mercaptopurine treatment in UC.37 In 1972 the immunosuppressive effect of cyclosporine was discovered and 12 years later in a report from Gupta et al cyclosporine was proven valuable in treatment of UC.38 In contrast to immunosuppressant treatment in Crohn’s disease, reports during the years have shown diverging results in UC patients. However, in refractory and non cortisone responding disease immunosuppressants have a place in the treatment arsenal as for maintenance treatment in severe disease.

Biological therapy. The most recent therapy in UC is TNF–α blockade. In 1994 Elliot et al tested infliximab, a monoclonal antibody against TNF-α in patients with RA. The study showed evidence that specific cytokine blockade can be effective in human inflammatory disease.39 In 1999 infliximab was tested for UC.40 The most recent multicenter studies, the active UC trials (ACT1 and ACT2) have shown infliximab to be efficacious in treating UC resistant to standard therapy.41

Probiotics have been shown to prevent stress induced intestinal translocation and increased permeability in a rodent model.42 In pouchitis a clear therapeutic effect has been demonstrated and is recommended as treatment by the European Crohn’s and Colitis Organisation (ECCO).43 In treatment of colitis, there are ongoing randomized studies of the benefit of probiotics.44,45
2. BACKGROUND TO THE STUDY

Structure of the large intestine

Gross anatomy

The human large intestine, about 1.5 meters long, extends from the cecum in the right iliac fossa to the anus in the perineum. It is divided into colon and rectum. The colon consists of the ascending, transverse, descending, and sigmoid portions. Mostly the colon is an intraperitoneal organ although parts of the descending colon and the rectum are located in the retroperitoneum. The superior mesenteric artery supplies the ascending and transverse colon while the descending and sigmoid colon as well as the upper part of the rectum are supplied by the inferior mesenteric artery. Through the arcade vessel of Riolan, the superior and inferior mesenteric arteries Anastomose. The rectum is supplied by branches of the internal iliac artery bilaterally through the middle and inferior rectal arteries. Blood is drained from the colon by the superior and inferior mesenteric veins into the portal vein. The middle and lower part of the rectum are drained by the rectal venous plexus into the internal iliac veins and thereby into the systemic venous system and not the portal system. Lymphatic vessels that follow the colonic and rectal arteries supply lymphatic drainage of the different colonic and rectal segments.

Figure 1. The different segments of human colon and rectum

Innervation of the right part of the colon by the parasympathetic nervous system is derived from the vagal nerve via the celiac superior mesenteric ganglia. Parasympathetic nerves to the transverse colon following the right and middle
into the blood. Amounts of vitamins, especially vitamin K and vitamin B, for colonic absorption cell lining of the colon as nourishment. In addition, the bacteria produce small indigestible carbohydrates (dietary fibres) are fermented into nutrients for the colonic commensal flora and their hosts is both mutualistic and symbiotic so that rather small group of bacterial species. The relationship between the human flora in the colon and 60% of the mass of faeces. Most of the bacteria belong to a species of bacteria, fungi and protozoa, however, bacteria make up most of the colonic barrier.54 Lymphatic tissues are found in the mucosa just are present adjacent to the epithelium covering the dome-like structure of the follicles that may extend through the muscularis mucosae to submucosa. M-cells and macrophages. Lymphocytes may be dispersed or arranged in lymphoid tissues that may extend through the muscularis mucosae and are surrounded by the lamina propria containing plasma cells, T lymphocytes, mast cells, fibroblasts, eosinophils and macrophages. Lymphocytes may be dispersed or arranged in lymphoid follicles that may extend through the muscularis mucosae to submucosa. M-cells are present adjacent to the epithelium covering the dome-like structure of the colonic lymphoid follicle.46 Lymphatic tissues are found in the mucosa just adjacent to the muscularis mucosae. The submucosa contains Meissner's plexus of ganglion cells and nerve fibres while Auerbach's plexus are situated between the inner circular layer and the outer three striped longitudinal layers of muscle, the teniae. The outermost part of the colonic bowel wall consist of serosa and pericolic fat

**Microscopic anatomy**

The large intestinal wall consists of three main tissue structures: mucosal, submucosal and muscle layers. The mucosa can further be divided into epithelium, lamina propria and muscularis mucosa. The colonic epithelium is composed of a mixture of columnar absorptive cells, goblet cells and to a minor extent enterochromaffin cells covering the surface and extending into the crypts of Lieberkühn. Near the bottom of the crypt, stem cells giving rise to transit cells which differentiate during transfer upwards to the luminal surface. Lymphocytes, eosinophils, and apoptotic cells may be found in the surface epithelium, as well as endocrine cells and Paneth cells in the proximal colon and caecum. Crypts extend from the surface to the muscularis mucosae and are surrounded by the lamina propria containing plasma cells, T lymphocytes, mast cells, fibroblasts, eosinophils and macrophages. Lymphocytes may be dispersed or arranged in lymphoid follicles that may extend through the muscularis mucosae to submucosa. M-cells are present adjacent to the epithelium covering the dome-like structure of the colonic lymphoid follicle.46 Lymphatic tissues are found in the mucosa just adjacent to the muscularis mucosae. The submucosa contains Meissner's plexus of ganglion cells and nerve fibres while Auerbach's plexus are situated between the inner circular layer and the outer three striped longitudinal layers of muscle, the teniae. The outermost part of the colonic bowel wall consist of serosa and pericolic fat

**Function**

The main function of the large intestine is to absorb water, store faeces and gas, and to transport and expel the stool. The large intestine houses between 300-1000 species of bacteria, fungi and protozoa, however, bacteria make up most of the flora in the colon and 60% of the mass of faeces. Most of the bacteria belong to a rather small group of bacterial species. The relationship between the human colonic commensal flora and their hosts is both mutualistic and symbiotic so that indigestible carbohydrates (dietary fibres) are fermented into nutrients for the bacteria and the waste products, acetate, propionate, and butyrate, are used by the cell lining of the colon as nourishment. In addition, the bacteria produce small amounts of vitamins, especially vitamin K and vitamin B, for colonic absorption into the blood.
Colonic barrier function

Colonic barrier

The epithelium of the gut constitutes the most important barrier between the body and the external environment in animals and humans. The intestinal barrier performs the double function of both uptake and absorption of nutrients, water and ions as well as being a selective barrier against harmful food components, bacteria and other antigens. The normal intestinal barrier allows small amounts of antigens to cross the mucosa to interact with the innate and adaptive immune system, called immuno-sampling. The term “colonic barrier function” describes all the mechanisms involved in the barrier homeostasis, figure 2. It can, however, be divided into intrinsic and extrinsic elements of the barrier function. The intrinsic colonic barrier consists of the single-cell polarized epithelial layer with its junctional complexes and the defined trans-cellular uptake and transport mechanisms that regulate the diffusion, transport and uptake of various molecules. The extrinsic elements of the colonic barrier includes the lumen itself, with the mucosal and glycocalyx layer IgA, commensal bacteria and the lamina propria immuno cells. Other protective mechanisms of the extrinsic element are the intestinal motility, rapid intestinal repair restitution.47

Colonic epithelial cells

Colonocytes constitute together with goblet cells 95% of the large intestinal epithelial cells. On the apical border, the colonocytes are covered with the glycocalyx, large carbohydrates formed in a reticulum. Enzymes and protein necessary for digestion and absorption of nutrients are also present in the glycocalyx. The adjacent colonocytes are interconnected by the junctional complexes; TJ, adherence junctions and desmosomes. Further, the colonocytes are firmly attached to the basal lamina by hemidesmosomes.48

Goblet cells produce mucin and release it into the intestinal lumen. The mucins consists of glycosylated proteins that lubricate the stool and protect the colonic barrier.49

Enterochromaffin cells, the most abundant enteroendocrine cell in the colonic epithelium, synthesize and store serotonin. In response to luminal stimuli, or signalling from the enteric nervous system, enteroendocrine cells release different hormones, i.e. serotonin, secretin, neurotensin and somatostatin.50

Paneth cells are stimulated into secreting defensins, lysozyme, phospholipase A2 and TNF-α when exposed to bacteria or bacterial products such as lipopolysaccharide, muramyl dipeptide and lipid A. The Paneth cells are located in the base of the intestinal crypts where they prevent bacterial growth.51

M cells (microfold or membranous cells) are found in the colonic follicle-associated epithelium of the lymphoid nodes that have the unique ability to sample antigens from the lumen and deliver them via transcytosis to antigen presenting cells and lymphocytes located in pocket-like structures on the basolateral side.46;52;53 M cells are targeted by several pathogens for invasion of the colonic barrier.54
Figure 2.

**Lumen:** Bacteria and antigens are degraded by bile, gastric and pancreatic juice. Further, the commensal bacteria inhibit pathogenic bacteria by competition.

**Microclimate:** The unstirred water layer, mucus layer produced by the goblet cells and the immunoglobulins (secretory IgA).

**Epithelium:** The colonocytes, connected by the junctional complexes, with active and passive transport mechanisms and transport pores into and between the epithelial cells. The basal lamina.

**Lamina propria:** Cells of innate and acquired immunity. Enteric nervous system and hormones. The endothelium of the capillaries and lymphatics.

Figure 3. The junctional complex. Left: Transmission electron micrograph from human colon. Left: Stylistic drawing showing details in the junctional complex.
The junctional complex

The colonic epithelial barrier consists of neighbouring cells adjoined in a series of intercellular junctions commonly called the junctional complex which was first described by Farquhar and Palade. The junctional complex consists of tight junctions, adherence junctions, desmosomes and gap junctions, see figure 3.

**The tight junction** (TJ) complex consists of almost 40 distinct proteins or protein families. TJ are located at the most apical part of the lateral cell membrane forming a network of linking strands between the adjacent epithelial cells constituting a paracellular barrier for passage of ions and molecules, see figure 4. The molecular structures of the TJ consist of:

- transmembrane proteins occludin and claudin.
- cytoplasmatic plaque proteins and adjacent protein structures.
- F-actin filaments of the cytoskeleton.

TJ appear as “kissing sites” in the plasma membrane shown in electron microscopy. The kissing sites are constituted of the transmembrane proteins claudins, occludin and JAM. The claudins consist of a tetraspan strand of protein. There are at least 24 gene variants coding for different claudins, which may form the basis for the different properties of the paracellular pathway in various epithelia. Occludin is also a tetraspan protein strand but does not have any known homologues. In contrast to claudin, the function of occludin is still unknown. The interface between the transmembrane proteins and the cytoplasmatic components of the TJ is formed by a set of scaffolding proteins that constitute the cytoplasmatic plaque. These have the ability to bind the C-terminal of transmembrane proteins i.e. claudins and occludin. Scaffolding proteins include the MAGUK (membrane associated guanylate kinase protein family) ZO-1, -2 and -3, other MAGUK relatives and polarity proteins. The transmembrane proteins occludin, claudins and JAM bind to the scaffolding proteins. JAM also play a role in immune cell trafficking across the epithelium. Finally the kinases, aPKC, c-Src and c-Yes binds to different proteins in the cytoplasmatic plaque and phosphorylates different proteins upon intracellular signalling.

TJ have a gate-like functionality, through which diffusion of solutes are rate limited, as well as a fence-like barrier separating lipids and protein components of the apical and basolateral cell membranes. These TJ functions seem to be regulated in different ways. Regarding the TJ gate function, there is both a size- and a charge-selectivity for ions and molecules, where positively charged molecules and ions diffuse more easily. There is convincing evidence that the composition of the claudins in various epithelia constitutes the basis for differences in size, charge and conductance properties of the paracellular pathway. Intracellular second messengers, such as cAMP, phospholipase C, protein kinase C, calmodulin and G-proteins regulates the gate function mainly via effects on components of the cytoskeleton.
Occludin the scaffolding proteins ZO-1 and cingulin and terminates on the plasma membrane at kissing sites within the TJ. The F-actin binds directly to the junction, called perijunctional actin-myosin ring. F-actin microfilaments projecting from the ring interface at kissing sites within the TJ. A belt of actin and myosin filaments circumscribes the apical part of the lateral cell plasma membrane. F-actin also constitutes an important structure of the microvilli. Actin forms a three-dimensional reticulum surrounding the cell interior close to the two isoforms, filamentous (F-) actin and globular (G-) actin. By cross-linking F-actin microfilaments contain the contractile proteins, actin and myosin. It is present in absorptive cells, microtubuli are important for endocytosis and transcytosis. Intermediate filaments are polymers of fibrous proteins that resist stretching and are important for cellular structure. Further, intermediate filaments link adjacent cells together via the desmosomes. Microfilaments contain the contractile proteins, actin and myosin. It is present in two isoforms, filamentous (F-) actin and globular (G-) actin. By cross-linking F-actin forms a three-dimensional reticulum surrounding the cell interior close to the plasma membrane. F-actin also constitutes an important structure of the microvilli. A belt of actin and myosin filaments circumscribes the apical part of the lateral cell membrane and encircles the epithelial cell just below the TJ at the adherence junction, called perijunctional actin-myosin ring. F-actin microfilaments projecting from the ring interface at kissing sites within the TJ. The F-actin binds directly to the scaffolding proteins ZO-1 and cingulin and terminates on the plasma membrane directly at the contacts and participates in the regulation of TJ barrier.

Figure 4. The tight junction complex

The cytoskeleton is a dynamic complex of protein filaments which is necessary for a variety of cellular functions such as mitosis, motility, cell polarity and changes in shape. The cytoskeleton consists of three major types of proteins, microtubules, intermediate filaments and microfilaments. In absorptive cells, microtubuli are important for endocytosis and transcytosis. Intermediate filaments are polymers of fibrous proteins that resist stretching and are important for cellular structure. Further, intermediate filaments link adjacent cells together via the desmosomes. Microfilaments contain the contractile proteins, actin and myosin. It is present in two isoforms, filamentous (F-) actin and globular (G-) actin. By cross-linking F-actin forms a three-dimensional reticulum surrounding the cell interior close to the plasma membrane. F-actin also constitutes an important structure of the microvilli. A belt of actin and myosin filaments circumscribes the apical part of the lateral cell membrane and encircles the epithelial cell just below the TJ at the adherence junction, called perijunctional actin-myosin ring. F-actin microfilaments projecting from the ring interface at kissing sites within the TJ. The F-actin binds directly to the scaffolding proteins ZO-1 and cingulin and terminates on the plasma membrane directly at the contacts and participates in the regulation of TJ barrier. Regulation of TJ permeability via the perijunctional actin-myosin ring is beginning to be understood. Induced activation of MLCK causes aggregation of perijunctional microfilaments and contraction of the perijunctional actin-myosin ring which, in turn, leads to a pulse-string-type contraction of the cell membrane and a centripetal-tension-generated-opening of the intestinal TJ barrier. TNF-α, known to increase TJ permeability in IBD, increases MLCK activity via activation of NF-κB, which leads to increased transcription and expression of MLCK and,
hence, MLCK–activated contraction of the perijunctional ring. Moreover, TNF-α-induced increase in TJ permeability can be inhibited by the anti-inflammatory drug prednisolone by suppressing the TNF-α induced increase in MLCK gene activity. In UC, increased production of interleukin 13 elevates claudin 2 protein expression in TJ which partly explains the impaired epithelial barrier function.

Furthermore, cytokines can induce disassembly of the apical junctional complex by promoting differential endocytosis of component proteins. For example, real-time assessment of TJ structure by fluorescent fusion constructs of TJ proteins showed that occludin can alter the paracellular permeability by a myosin light chain kinase (MLCK) dependent caveolae-mediated endocytosis of occludin. How the endocytosis of occludin is regulated is still unknown.

**Adherence junctions** are situated below the TJ and consist of cadherins, a Ca²⁺ dependent protein. Further, cadherin-mediates cell to cell adhesion and is important for development and maintenance of epithelial tissue integrity. Through intracellular domains, cadherins associate with molecules of the Armadillo superfamily including β-catenin, which links them to the actin cytoskeleton via the α-catenin bridge.

**Desmosomes** are intercellular junctions, which provide mechanical integrity to the intestinal epithelium by anchoring intermediate filaments to sites of strong adhesion. Desmosome assembly and disassembly is regulated by calcium, kinase/phosphatase activity, proteolytic processing, and cross talk with adherence junctions. Regulation of transcripts of desmosomal cadherins specifies the distinct composition, expression and tailoring desmosome functions within different cells.

**Gap junctions** are composed of two aligned hemichannels, called connexons that are assembled out of six connexin molecules. The gap junctions provide low-resistance channels between cells, allowing easy and rapid spread of electrical currents and biochemical messengers via small molecules. Because of heterogeneity in connexin molecules, the function of the connexons provide different conductance, probabilities of opening, voltage dependencies of opening probability, and susceptibilities to closure by different agents.
Uptake and transport

Endocytosis

Endocytosis is a diverse set of processes used by the cell to internalize specialized regions of the plasma membrane as well as small amounts of extracellular fluid, polar macromolecules and larger structures such as whole bacteria and apoptotic cells, structures that can not pass through the hydrophobic plasma membrane or through the junctional complex. Most studies of intestinal epithelial endocytosis have been conducted on small intestinal villous and follicle associated epithelium. However endocytosis occurs in the colonic enterocytes as well as colonic M-cells. Endocytosis occurs by multiple mechanisms that fall into two broad categories, phagocytosis and pinocytosis. Phagocytosis is typically restricted to specialized cells, whereas pinocytosis occurs in all cells by at least four basic mechanisms: macropinocytosis, clathrin-mediated endocytosis , caveolae-mediated endocytosis, and clathrin- and caveolae independent endocytosis, figure 5.

Clathrin mediated endocytosis is the best studied mechanism. It is a receptor-ligand mediated pinocytosis. The high affinity receptors are trans-membranous located and bind with high affinity to the cargo molecule. The receptor-ligand complex concentrates into coated pits which are formed of clathrin and other assembly proteins e.g. assembly-protein-2 (AP-2) adaptor complexes, and the dynamin GTP:ase. The coated pit invaginates to form a vesicle and then pinches off as an endocytotic vesicle with the receptor-ligand complex inside, see figure 5. Clathrin mediated endocytosis is used in cell homeostasis to regulate internalization of ion pumps, ion channels and in regulation of the intestinal barrier in IBD by clathrin mediated endocytosis and internalisation of apical junctional complex. Further, clathrin mediated endocytosis is important in uptake of essential nutrients for instance low density lipoprotein (LDL) and transferrin.

Caveolae mediated endocytosis involves caveolae -flask-shaped- invaginations of the cellular membrane. Caveolae defines cholesterol and sphingolipid-rich microdomains in the plasma membrane in which many signalling molecules and membrane transporters are concentrated. Caveolin, a protein that binds cholesterol, inserts as a loop into the inner leaflet of the plasma membrane and forms a caveolin coat on the surface of membrane invagination. Inside the caveolae, receptors bind the cargo protein and by a signalling cascade, involving the G-protein and kinases, the caveolae is internalized. Transcellular transport of albumin is known to use the caveolae route. Further, regulation of TJ proteins by caveolae uptake and internalisation have been shown and whole virus particles and viral enterotoxins use caveolae for amplification of viral morphogenesis.

Clathrin and caveolin independent endocytosis constitutes pinocytosis by small rafts of individual lipid composition which attract specific membrane proteins and/or glycolipids. The rafts diffuse freely on the cell surface and can be captured by and internalised by any endocytic vesicle. Clathrin independent
endocytosis occurs in neuroendocrine cells and neurons enabling rapid recovery of membrane proteins after stimulated secretion. Further, clathrin and caveolin independent endocytosis is also seen in cellular bacterial toxin uptake. The mechanisms of internalisation in clathrin and caveolin independent endocytosis are poorly understood.

![Figure 5. Possible routes for permeation across the intestinal epithelium. A: Transcellular route (lipophilic and small hydrophilic compounds). B: Paracellular route (larger hydrophilic compounds). C: Transcellular via pores. D: Active carrier-mediated absorption (nutrients, e.g. glucose) E: Pinocytosis and transcytosis (Clathrin, caveolin dependent/independent). F: Phagocytosis and/or macropinocytosis.]

**Phagocytosis.** Larger particles, microbes (i.e. bacteria fungi and viruses) and remnants of dead cells are taken up via adsorptive endocytosis. Receptors for specific antigen uptake are exposed on the cell surface. In uptake of microbes, the Fc-receptors recognize and attach to the antibodies bound to surface antigens on the microbe. A signalling cascade triggers formation of cell surface extensions around the antibody coated pathogen. Inflammatory responses are activated and the microbe is destroyed inside the phagosome. Later, regurgitated microbe peptides are presented on the cellular surface to elicit a response from the immune system. Phagocytosis is seen primarily in macrophages, monocytes and neutrophils but bacterial internalisation in enterocytes has been shown. The toll-like-receptor-4 (TLR-4) is required for this process. Phagocytosis is also relevant for the non-specific uptake of luminal dietary and bacterial antigens and is more common in M-cells. The process is triggered by secreted bacterial products.

**Macropinocytosis** resembles with phagocytosis but is not receptor mediated and does not enclose the cell surface extensions around a ligand-coated particle but rather collapse onto and fuse with the plasma membrane to generate endocytic vesicles called macropinosomes. Colonic enterocytes, M-cells and especially dendritic cells are triggered into prolonged macropinocytic activity by antigen activation. This results in large samples of the extracellular milieu for immunosurveillance. The protein antigen HRP is known to be taken up by this
mechanism. In addition, viral antigens induce macropinocytosis in order to amplify viral uptake.

**Transcytosis**

Transcytosis is the process by which various macromolecules are transported inside an endosome across the interior of the cell. The intestinal epithelium traffic is either back to the same cell membrane, in the apical to basolateral direction or in the opposite direction. At the plasma membrane, proteins can either remain at the cell surface or be rapidly internalized into endosomes. Endosomes are transported to either the apical or basolateral sorting compartments now entitled apical or basolateral early endosomes. Inside the enterocyte, endosomal cargo proteins are sorted. The early endosome can now either recycle to the plasma membrane with or without releasing the endocytotic cargo protein or merge with lysosomes or be transported from one cell membrane to another i.e. apical-basolateral. The proper operation of these transport routes requires that several important sorting decisions be made along the way. The decisions are governed by a complex system of sorting signals in the cargo proteins and molecular machinery that recognizes those signals and delivers the proteins to their intended destinations. Transcytosis occurs in one of three ways in intestinal enterocytes.

a) Vesicles internalize from the apical membrane and form endosomes. In the sorting compartment they merge and form apical early endosomes and are recycled back to the apical membrane, with or without cytoplasmic release of their cargo. The cargo (protein, virus or particle), bound to the internalised receptor, is normally released into the cytoplasm while the receptor is recycled back to the cell membrane. However, large molecules i.e. peptides and proteins may be degraded since diffusion progresses slowly through the cytoplasm.

b) Early endosomes are delivered to late endosomes and then merged with lysosomes. The cargo is degraded and the endosomal receptor proteins recycle back to the cell surface.

c) Endosomes formed in the apical part of the cell membrane may be transported to the basolateral sorting compartments and merge with basolateral early endosomes.

It is known that enterocytes not only transport internalised antigens. During chronic bowel inflammatory diseases i.e. IBD and celiac disease, enterocytes in fact act as non-professional antigen-presenting cells and promote inflammation.
Regulation of endocytosis and transcytosis

Regulation of endocytosis and transcytosis is not fully elucidated. Different types of challenges to the host lead to increased uptake of luminal antigens. Bacterial exposure leads to enhanced uptake and transport across the intestinal epithelium. Bacterial stimulation also induces production of pro-inflammatory cytokines. Transepithelial HRP uptake has been shown due to TNF-α in intestinal epithelial cell culture, and increased transcytosis of HRP could be correlated to TNF-α mRNA levels in the underlying mucosal tissue. Further, cold and restraint stress and chronic stress leads to increased transcellular uptake of HRP. In vitro studies show increased uptake of marker molecules via the transcellular route in intestinal epithelium challenged with the putative stress mediators acetylcholine (Ach) and corticotropin releasing hormone (CRH) and its antagonists.

Studies of intestinal permeability

Studies of intestinal permeability in humans have been carried out at different levels of complexity.

In vivo measurements are the most commonly used techniques in humans. The test substances, usually a non-degradable hydrophilic marker molecule, is given orally and the clearance of the marker is measured in blood or in urine. These studies are easy to perform and physiological but give limited information on passage routes and mechanisms involved in barrier function.

In situ techniques were first described by Fordtran et al (1961). Intestinal permeability was studied by using a triple lumen nasogastric tube for infusion of hypertonic solutes and for collection of intestinal juices. In the regional perfusion techniques (Loc-I-Gut®, Loc-I-Col®) a certain region of the small and large intestine could be studied. However, using tubes, either orally or rectally, is unpleasant for the test subject and the test is time-consuming. Further, the method measures only the disappearance of marker molecules from the perfusate rather than the actual epithelial uptake.

In vitro studies use tissue from either patient subjects such as resected specimens or biopsies, further, cell culture of colon cancer cell lines such as Caco-2, HT-29, and T84 are used. These cell lines differentiate to a polar epithelium upon culture, however, the epithelial properties are different from normal epithelium. Both the intestinal samples and cell cultures can be studied in a diffusion chamber where the tissue constitutes the barrier between the luminal and baso-lateral side of the epithelium. The most used chamber technique is the Ussing chamber. The Ussing chamber technique allows mechanistic studies at the cellular level without the limitations of using harmful markers or harmful epithelial modulator substances. Major drawbacks in all in vitro techniques are the absence of normal circulation and innervation.
The Ussing chamber

The Ussing chamber was invented by the Danish physiologists Ussing, H.H and Zerahn, K. In their first publication in 1951 they showed that active transport of sodium was the source of electric current in the short-circuited isolated frog skin. However, the original method described by Ussing was complicated and was subsequently modified in 1988 by Grass and Sweetana. The modified Ussing chamber consists of two half chambers with the tissue sample mounted as a semi-permeable membrane between the halves. The two reservoirs on each side of the tissue sample is filled with buffer and continuously oxygenated. Thanks to the gas flow the buffer is continuously stirred, which reduces the thickness of the unstirred water layer. With a heater block system the tissue samples and solutions are kept at 37°C. Marker solutions and modulator substances can be applied on the apical or baso-lateral side of the tissue. Samples for marker permeation may be drawn from either side of the chamber reservoirs.

Figure 6. Left: Biopsy forceps during sigmoidoscopy. The biopsy is mounted on the plastic slide in Ussing half chamber. Right: Assembled Ussing chamber coupled to gas flow and electronic equipment.

Electrophysiology. A transepithelial potential difference (PD) over a transporting polar epithelium is the reflection of all electrogenic pump activities in the epithelial membrane. These consist mainly of Na⁺/K⁺-ATP:ases and the passive permeation of ions through channels apically and baso-laterally and ion flux over the TJ. Further, the PD can be divided into current and resistance, according to Ohm’s law. Short circuit current (Isc) denotes a current where PD =0 and reflects
the summation of all ion pump activity. In the mucosal sample not only an epithelial cell lining is present but also subepithelial tissue. The total transmural resistance over the sample in the Ussing chamber is built up of: a. apical and b. baso-lateral epithelial cell membrane, c. resistance in TJ and d. subepithelial resistance. The \((a+b)\) = transcellular resistance, \((a+b+c)\) = epithelial resistance and \((a+b+c+d)\) = transepithelial resistance (TER) and reflects the resistance against ions passing the epithelial and subepithelial routes. With the square current technique, measurements of the impedance is introduced and the TER can further be calculated and divided into epithelial \((a+b+c)\) and subepithelial resistance \((d)\). In the original Ussing method the epithelium was short-circuited by a voltage clamp and the current was measured by an amperemeter. Later, an analogue/digital (A/D D/A) board was invented and linked to a computer for electrophysiological measurements. The present setup for electrophysiological measurements in the Ussing chamber experiments are reviewed in the methods chapter.

**Probes.** The Ussing chamber technique for in vitro studies of epithelial function is well established for studies of intestinal ion transport, drug absorption, and permeability to various-size marker molecules and antigens in gut mucosa (for reviews, see references 141-145). \(^{51}\)Cr-EDTA, \(^{14}\)C-mannitol, FITC-dextran, inulin and different sized polyethylene glycols (PEG) are examples of small water soluble molecules with a predominantly paracellular route used in Ussing chamber studies. \(^{146-150}\) In studies of transcellular uptake mechanisms, ovalbumin and HRP have been used. \(^{25;151;152}\) HRP type 4 is a 44kDa, single chain polypeptide. The main advantages of HRP include the known uptake mechanisms through macropinocytosis. Further, it is a protein antigen, easy to stain and quantify with enzymatic methods. The main drawback of HRP is the risk of contamination of buffer samples by other peroxidases which are present in the studied tissue i.e. mast cell granules and on the hands of the researcher. Erroneous peroxidases will interfere with the quantification process. \(^{153}\)

**Specimens.** In vitro studies of barrier function in human intestine have mainly been limited to intestinal specimens from patients undergoing GI-surgery. \(^{24;25;146;154-156}\) The use of surgical specimens does, however, have some obvious drawbacks. The patients have possible alterations in physiology due to medication, surgical stress, malnutrition and other factors before and during surgery that are known to influence the mucosal barrier. \(^{126;157}\) Furthermore it is difficult to find adequate control groups as, by definition, patients in need of bowel surgery are not healthy. In recent years, endoscopic biopsies of human intestine have been used to assess ion secretion as well as TJ permeability to ions and mono- and disaccharides. \(^{158-161}\) Only two studies of intestinal mucosal permeability to protein antigens in endoscopic biopsies have been reported. \(^{18;162}\) There are, however, no studies on viability and evaluation of the biopsy technique in macromolecular permeability experiments.
Stress

Psychological stress

Stress is a normal component of life and adequate responses to different stressful situations are required for survival. However, there are large individual variations in the possibility to cope with stressful life events. Stress has been defined as a disruption of homeostasis, which may be triggered by a physical (real) or psychological (perceived) stimulus that produces mental or physiological reactions which might lead to illness. Under normal circumstances physiological stress reactions will be dampened in an adaptive response. However, in individuals susceptible to stress a maladaptive response may become harmful. Pathologic stress reactions are often associated with prolonged stressful daily life events for example unemployment, family losses or conflicts, severe illness in a near relative, etc. Despite different origins of stress events, the triggered reaction to maintain homeostasis follows the same pattern. The stress response consists of behavioural and autonomic reactions due to the hypothalamic-pituitary-axis (HPA) with release of various stress hormones in the brain and further into the autonomic nervous system, see figure 7. The association between psychological stress and the development of chronic gastrointestinal inconveniences and disease remains a controversial issue but has for a long time been described by patients. Barclay and Turnberg have shown that psychological stress, induced by dichotomous listening and cold-induced hand pain reduced water absorption and transformed sodium and chloride absorption into secretion in the jejunum. These effects were inhibited by atropine (At), suggesting the involvement of cholinergic neurons (Ach). Further, these effects were associated with luminal release of cell contents histamine and tryptase from mast cells. These results suggested an obvious interaction between psychological stress and intestinal mucosal function.

Pathophysiology. Persistent stress and stressful life events have been shown to increase the risk for exacerbations of UC, whereas the epidemiological evidence of an association between stress and Crohn’s disease is less clear. In patients with irritable bowel syndrome (IBS) there is a relationship between stress and symptom severity (for review, see Söderholm), and stressful life events appear to increase the risk of developing post-infectious IBS. It is established that the mucosal barrier to environmental antigens is of importance in the disease process in IBD. Patients with Crohn’s disease exhibit an increased intestinal permeability, and display strong immune reactions to dietary antigens and bacteria. Furthermore, in IBS patients increased permeability persisted for several months after gastroenteritis due to post-infectious immune activation, and an increased number of intestinal mucosal mast cells have been demonstrated in IBS mucosa. Psychological stress is also believed to cause alterations in the gut barrier function with increased intestinal ion secretion changes in humans and increased transepithelial uptake of antigens, bacterial attachment/penetration, mast cell hyperplasia and inflammatory cell infiltration in animals. Chronic stress also may induce exacerbation of mucosal inflammation in rodents.
Stress mediators. Rodents exposed to acute stress show increased mucosal permeability to macromolecules via mechanisms that mainly involve CRH, mast cells and cholinergic nerves. Treatment with the non-specific muscarinic Ach antagonist, atropine, prevents stress-induced increase of HRP uptake in rat jejunum, suggesting cholinergic involvement in the regulation of HRP uptake in stress. Gareau et al have recently shown increased colonic uptake of HRP as an effect of increased cholinergic nerve activity in rat pups due to maternal separation stress. CRH has also been implicated as an important mediator of stress-induced abnormalities in mucosal function in animal models. Stress-induced changes in rat colonic epithelial function can be inhibited by CRH receptor antagonist. In addition, injection of CRH mimics stress-induced changes in colonic function regarding mucin release, ion secretion and permeability (paracellular as well as transcellular). Mast cell stabilizer have been shown to abolish the CRH-induced changes. Different CRH receptor subtypes have been found to be expressed in isolated lamina propria mononuclear cells from human colonic mucosa. Selective CRH-R1 antagonists have been shown to inhibit stress effects on intestinal motor function in rats, whereas blocking of the CRH-R2 did not. Furthermore, Barreau et al reported in adult rats previously exposed to neonatal maternal deprivation that CRH promoted colonic mast cell release of nerve growth factor (NGF) via the CRH-R1 receptor and as a result, gut paracellular permeability were increased. However, to date, the relative importance of the CRH receptors in human intestinal mucosal function has not been reported.
Neuroimmune regulation of the colonic mucosa

The peripheral enteric nervous system (ENS) is constituted of mainly two defined plexi, the myenteric plexus (Auerbach) which is located between the inner circular and outer longitudinal muscle layer and the submucosal plexus (Meissner) which is located in the subepithelial space. These plexi consist of sensory, inter-, secretomotor and vasodilator neurons. The myenteric plexus primarily operates the gastrointestinal movements and the submucosal plexus controls the secretion and blood flow of the mucosa. Secretion and absorption are controlled by Ach, norepinephrine and a variety of different efferent neurons and neurotransmitters in the ENS. The epithelial process is regulated by an intracellular increase of Ca\(^{2+}\) and cAMP, by formation of inositol-3-phosphate and activation of adenylate cyclase which affect the activity or membrane abundance or both of various transport proteins.\(^{191-193}\) The various processes in the mucosa, intestinal smooth muscle and visceral vasculature are meticulously controlled by the ENS, and are targeted by axons of both sympathetic and parasympathetic neurons. Signalling from the subepithelial inflammatory cells, e.g. polymorphonuclear leucocytes, lymphocytes, macrophages and mast cells,\(^{194;195}\) to the ENS establishes an important line of defence against foreign invasion of hazardous substances and microbes in the intestinal mucosa. Afferent signals from the inflammatory cells, predominantly mast cells, are spread to and by the ENS via a combined paracrine and immunoneural axonal reflex by submucosal inter-neurons resulting in further mast cell activation, mucosal secretion and motor propulsion.\(^{196}\) Paracrine signalling between inflammatory cells and the ENS have mostly been studied in mast cells, however, all immune/inflammatory cells are putative sources of signalling.\(^{197;198}\) By activation/degranulation, mast cells release mediators with neuropharmacological actions on neurons in the ENS such as, histamine, IL-6, 5-HT and PAF. Antigens or other activating signals to the mast cell trigger an activation/degranulation and release of mediators which become the paracrine signal. The ENS recognises the signal and starts a defensive program to eliminate the threat, for instance diarrhoea. Further, mast cells and the central nervous system (CNS) are linked via the brain-gut connection. Mast cells may activate/degranulate due to psychological stress via CNS efferent vagal signalling, which activates the “alarm program” in the ENS. This will produce the same symptoms as antigen evoked degranulation of mast cells such as activating the secretomotor signals resulting in diarrhoea.

3. AIMS OF THE THESIS

The aim of this thesis was to investigate the colonic barrier function of healthy volunteers and in UC patients with special reference to neuro-immunological regulation by the putative stress mediators CRH and Ach. To enable permeability studies in normal subjects a technique using endoscopic biopsies in the Ussing chamber was developed and evaluated.

The specific aims of the separate studies were to:

I. Develop and evaluate the use of endoscopic biopsies from sigmoid colon in Ussing chambers as a tool for studies of permeability to macromolecules with antigenic potential.

II. Characterize the peripheral effects of CRH on the mucosal barrier function in normal human colon as well as the mechanisms involved in the CRH-induced transcellular hyper-permeability.

III. Investigate the role of cholinergic signalling and the involvement of subepithelial mast cells in the regulation of para- and transcellular permeability and ion secretion in normal human colonic mucosa.

IV. Elucidate macromolecular permeability and ion secretion in the macroscopically non-inflamed colon of ulcerative colitis patients. Further, to characterize the link between cholinergic and CRH signalling, eosinophilic leucocytes and mast cells in transcellular permeability to protein antigens.
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4. SUBJECTS AND METHODOLOGY

Volunteers, patients endoscopy and ethics

Healthy volunteers (Papers I-IV)

Ninety-one healthy volunteers were included in the four studies, 42 women and 49 men, with a median age of 23 yrs (range 20-75). Inclusion criteria were no medication, non smoking, no history of IBS or IBD, and no family history of IBS/IBD. No volunteers were excluded due to unexpected illness at examination or technical problems at sigmoidoscopy. Routine clinical histology of sigmoid biopsies was normal in all subjects.

Patients (Paper I and IV)

In addition to biopsies from healthy volunteers in the first study, resected specimens from sigmoid colon were collected from three patients who underwent rectal cancer surgery. Comparisons were done between biopsies and specimens with larger exposed area in the Ussing chamber. The UC group in paper IV consisted of 15 patients who were scheduled for annual surveillance colonoscopy. Inclusion criteria were UC diagnosis based on reviewed histo-pathology report specimen and patient data registration in the Swedish IBD registry (SWIBREG). Only patients in remission from UC and a history of no other severe illness were included. Exclusion criteria were colitis of other origin than UC, i.e. indeterminate colitis or Crohn’s colitis (one patient was excluded due to histopathology). The research biopsies were taken during planned surveillance colonoscopy. The median age was 51 years (range 21-78) and there were 5 women and 10 men included.

Endoscopy

Healthy volunteer (papers I-IV) Preparation was carried out using the sodium-docusate-sorbitol laxative enema (Klyx®, Ferring, Copenhagen, Denmark) at 12 and 2 hours before exam. Only clear drinks were allowed after the first enema until exam. Flexible sigmoidoscopy was performed and biopsies were taken at the 20-30 cm level in the sigmoid colon.

Ulcerative colitis patients (paper IV) Bowel preparation was performed the day before exam with the orally given macrogol laxative (Laxabon®, BioPhausia AB, Stockholm Sweden) After laxative treatment only clear drinks were allowed until exam. Colonoscopy was performed in our outpatient ward. Biopsies were taken during the first part of exam according to our lab protocol at the median level of 30cm (range 20-50 cm) from anal verge.
Subjects and methodology

Ethics

The study protocols involving healthy volunteers and patients were approved by the Ethical Committee, Faculty of Health Sciences, Linköping Sweden and was conducted according to the Declaration of Helsinki.

Methodological considerations I

Age: The peak incidence of UC is between the ages of 15 and 35 years but has been reported in every decade of life. In our studies the healthy volunteers were in median 25 years old, the same age as the age of onset in the UC group. However, the UC patients in the fourth study were substantially older (median 51yrs) than the control group (median 23 yrs). Differences in ages between study groups could possibly influence the results. However, there is no support in the literature for altered macromolecular permeability in adults due to age. In our studies there was no correlation between age and HRP permeability ($r^2 =0.02$, $p= n.s$, $n=55$ volunteers)

Further, the two groups, healthy volunteers and UC patients, differ in medication. None in the control group were on any medication. In contrast, all but one in the UC group was on some sort of medication for UC which is known to influence colonic inflammation and reduce permeability. However, we found increased macromolecular permeability in the UC patients in spite of medication.

Preparation: There were also dissimilarities between healthy volunteers and UC patients in preparation for colonoscopy. The UC patient group were all prepared by orally given laxative Laxabon, known to induce colonic inflammatory changes with increase of subepithelial inflammatory cells in colon. This is not well studied for the rectal enema Klyx®. However, in the histo-pathology reviews no inflammatory changes were seen in biopsies from healthy volunteers. In the UC patients, inflammatory mucosal changes were related to UC. There is however a risk for confounding factors when comparison is made between groups not prepared in the same way.
Using chamber experiments

Biopsy preparation and mounting

In each subject 15-20 biopsies were taken with a biopsy forceps without a central lance (CBF 2.5-230, Cook Sweden AB, Askim, Sweden) from macroscopically normal mucosa in the mid section of the sigmoid colon (20-30 cm level in healthy volunteers and 20-50 cm level in UC patients). The biopsies were put in cold oxygenated modified Krebs-Ringer bicarbonate buffer and transported to our laboratory within 20 minutes. The biopsies were mounted in modified 1.5-ml Ussing chambers (Grass & Sweetana,138 Harvard apparatus Inc. Holliston, MA, USA) with a diameter of 9 mm opening and reduced to an exposed tissue area of 1.76 mm², using a technique previously described by Bijlsma et al.138 The biopsies were mounted between two 0.4 mm polyester films with a diameter of 1.5 mm opening with rounded edges. The flexibility of the films reduced squeezing of the tissues at the border of the openings to minimise edge damage. The surface of the polyester was roughened with fine abrasive paper to keep the biopsies in position. The polyester films were sealed together with inert silicone grease to prevent leakage between the polyester slides. After mounting, each half chamber was filled with 1.5 ml modified Krebs-Ringer bicarbonate buffer (KRB) with the mucosal and serosal side separated by the specimen. The modified KRB contained (mM) NaCl, 110.0;CaCl₂, 3.0; KCl, 5.5; KH₂PO₄, 1.4; NaHCO₃, 29.0; Na-Pyruvate, 5.7; Na-Fumarate, 7.0; Na-glutamate, 5.7 and glucose 13.4mM. The KRB was pH adjusted to 7.4 and equilibrated by O₂/CO₂ (95/5%) before use. The KRB solution was continuously oxygenated with O₂/CO₂ (95/5%) and stirred by the gas flow in the chambers and kept at 37°C with a heater block system. After a 40-minute equilibration period to achieve steady state conditions regarding PD, the KRB in the mucosal and serosal compartment were replaced with marker and modulator/vehicle solution respectively.

Viability

In order to evaluate the Ussing technique for endoscopic biopsies, it was important to examine the viability of the biopsies in the Ussing chamber system. As viability marker we used levels of ATP and lactate and the ability of the biopsy to uphold an electrical potential difference. Samples were therefore taken at endoscopy, at the time of mounting, and after different time points up to 120 minutes in chamber. A 2-mm diameter routine skin sample punch was used to dissect exposed mucosa free of surrounding flattened tissue. The samples were snap frozen in liquid nitrogen, stored at −70°C, and freeze-dried. ATP and lactate were extracted and measured with a fluoro-enzymatic method described and modified from Harris.203
Subjects and methodology

Permeability

To assess permeability to macromolecules across the colonic mucosa, horseradish peroxidase (HRP) and \textsuperscript{51}Cr-EDTA were used in all Ussing chamber experiments.

\textsuperscript{51}Cr-EDTA (Perkin Elmer, Boston, MA, USA; mw 340) with a specific activity of 126 Ci mmol\textsuperscript{-1} was added to a concentration of 1.11 mgL\textsuperscript{-1} (3.25\mu M). Permeation of \textsuperscript{51}Cr-EDTA was assessed by measuring the appearance of marker on the serosal side during the experiments. The radioactivity in 0.3 ml samples was counted for 600s in a gamma reader (1282 Compugamma LKB, Bromma Sweden). The apparent permeability coefficient \( P_{\text{app}} \) was calculated as follows;

\[
P_{\text{app}} \text{ (cm s}^{-1} \text{)} = \frac{(dC/dt)V}{(C_{t0}A)}
\]

\( dC/dt \) = change in submucosal concentration per unit time (molL\textsuperscript{-1}s\textsuperscript{-1})

\( V \) = volume of the chamber (cm\textsuperscript{3})

\( A \) = area of exposed sigmoid colon

\( C_{t0} \) = initial mucosal reservoir marker concentration (molL\textsuperscript{-1}).\textsuperscript{204}

**Horseradish Peroxidase type VI** (Sigma Chemical Co. St. Louis, MO, USA) was added on the mucosal side at a concentration of 10 \mu M. Samples from the serosal chamber at various time points were analysed with the QuantaBlu\textsuperscript{TM} Fluorogenic Peroxidase Substrate Kit (Pierce, Rockford, USA) according to a protocol developed at our laboratory.\textsuperscript{183} Samples were collected from the serosal side of the chambers and diluted in Krebs buffer with 0.02 % bovine serum albumin and 50 \mu l was transferred to dark micro titre plates. To each well, 100 \mu l of QuantaBlu\textsuperscript{TM} Working Solution (QuantaBlu\textsuperscript{TM} Stable Peroxidase Solution diluted 1:10 in QuantaBlu\textsuperscript{TM} Substrate solution) was added and the plate was incubated in a shaker at 300 rpm at room temperature. After 30 min, 100 \mu l of QuantaBlu\textsuperscript{TM} Stop Solution was added and 10 min later the absorbance of the coloured reaction product was measured at excitation wavelength 325 nm and emission wavelength 420 nm using a fluorimeter (Cary Eclipse, Varian, Victoria, Australia). Blanks were included in each analysis and all samples were run in duplicate and measured against a standard curve. The intra-assay variability coefficient was estimated to 8.6 % and the inter-assay variability coefficient to 9.3 %. HRP flux was presented as pmol/cm\textsuperscript{2}/h during steady state permeation in the 30-90 minute interval.

**Modulation.** In all papers modulation of mucosal permeability and electrophysiology were tested. The different agents are described in detail in table 1.

**Electrical measurements**

A four electrode system was used as previously described by Söderholm et al.\textsuperscript{205} One pair of Ag/Ag-electrodes (Ref 201, Radiometer, Copenhagen, Denmark) with 3M NaCl/2% agar bridges was used for measurements of PD and one pair of platinum electrodes for current passage. PD, TER and Isc were obtained as
described by Karlsson et al.\textsuperscript{206} The electrodes were coupled to an external six channel electronic unit with a voltage controlled current source. 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, USA). Every second minute, direct pulses of 1.5, -1.5, 3, -3 and 0 \( \mu \)A with duration of 235 ms were sent across the biopsy 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: \( U = PD + \text{TER} \times I \). The transepithelial resistance (TER) was obtained from the slope of the line, PD from the intersection of the voltage axis (when \( I = 0 \)) and the Isc is determined from the quotient PD/TER. In paper 1, PD was tested as a viability discriminator. There was a correlation between biopsies with a PD less negative than -0.5 mV after equilibration and leakage to macromolecules. Therefore, in all papers, biopsies with a PD > -0.5 mV have been excluded.

<table>
<thead>
<tr>
<th>Substance</th>
<th>Action</th>
<th>Conc. (molar)</th>
<th>Side of biopsy</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbachol</td>
<td>Muscarinic agonist</td>
<td>( 10^{-7} )</td>
<td>serosal</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td></td>
<td>( 10^{-6} )</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>CRH</td>
<td>CRH rec. agonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>II, IV</td>
</tr>
<tr>
<td>Sodium caprate</td>
<td>Mitochondrial impairment</td>
<td>( 10^{-5} )</td>
<td>mucosal</td>
<td>I</td>
</tr>
<tr>
<td>Atropine</td>
<td>Muscarinic rec. antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>III, IV</td>
</tr>
<tr>
<td>4-DAMP</td>
<td>Muscarinic rec. 3 antagonist</td>
<td>( 10^{-7} )</td>
<td>serosal</td>
<td>III</td>
</tr>
<tr>
<td>Hexametonium</td>
<td>Nicotinic rec. antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>III</td>
</tr>
<tr>
<td>( \alpha )-helical CRH(9-41)</td>
<td>CRH rec. antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>II, IV</td>
</tr>
<tr>
<td>Astressin</td>
<td>CRH rec. 1 antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>II</td>
</tr>
<tr>
<td>Antalarmin</td>
<td>CRH rec. 2 antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
<td>II</td>
</tr>
<tr>
<td>Lodoxamide</td>
<td>Mast cell stabilizer</td>
<td>( 10^{-5} )</td>
<td>serosal</td>
<td>II, III, IV</td>
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<tr>
<td>Lidocaine</td>
<td>Axonal Na\textsuperscript+ -channel antagonist</td>
<td>( 10^{-6} )</td>
<td>serosal</td>
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<tr>
<td>Tetrodotoxin</td>
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<td>( 10^{-6} )</td>
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</table>

Table 1. Antagonists were added 20 minutes before agonists or vehicle. To determine dose-response effects of Carbachol and CRH in Isc, TER and mucosal macromolecular uptake, biopsies were exposed to Cch and CRH at different concentrations. Sodium caprate (C10) vehicle, Ca\textsuperscript{2+} was omitted to avoid precipitation of the Ca\textsuperscript{2+}-salt of C10. Depletion of Ca\textsuperscript{2+} on the mucosal side does not, however, affect the integrity of epithelia as long as normal concentrations are maintained on the serosal side.\textsuperscript{205,207,208}
Methodological considerations II

**Viability:** *In vitro* techniques, like Ussing chambers, have limitations (e.g. the lack of circulation and nervous control) but it is necessary to use this approach for studies of mechanisms involved in human intestinal mucosal function. We have shown in the first paper that colonic biopsies have good viability in Ussing chambers, and can be used to study transcellular uptake of protein antigens and paracellular permeability to marker molecules. Moreover, the study showed that mucosal permeability as well as electrophysiological parameters can detect barrier alterations in our system.

**Edge leakage:** With a small tissue area exposed to the Ussing chamber the influence of edge damage on electrophysiological measurements increases, and our baseline values of Isc and TER suggest that some electrical edge leak may occur. However, changes in electrophysiology were monitored during modulation of the biopsies and the experimental system detected the minute changes in TER and Isc. In subsequent studies we showed that permeability in human intestinal mucosa was equal in the biopsy chambers and chambers for surgical specimens (exposed area 63.6 mm²), demonstrating that the possible electrical edge leak did not affect mucosal permeability to macromolecules figure 8. Inflamed human tissue is handicapped by an increase in subepithelial resistance, which measurements of Isc and TER overestimates. Epithelial resistances are therefore underestimated in our present setup, necessitating impedance analysis or square-pulse analysis for adequate assessment. We are currently establishing the square-pulse technique in our laboratory.

![Figure 8. Comparison of permeability to macromolecules between Ø 1.5 mm. modified Ussing camber with an exposed area of 1.76mm² and Ø 9 mm. conventional Ussing chamber with an exposed area of 63.6 mm².](image)

**Data scattering:** Another issue is the scattering of data in UC patients as well as in the healthy volunteers, which is higher than usually seen in experimental studies in animals. The studies were, however, done in a consecutive group of volunteering human subjects and UC patients with a very diverse genetic and environmental background. In spite of this, we found significant effects of neuro-immune modulation on HRP uptake and electrophysiology in the studied subjects.
Histology

Light microscopy

Viability and damage (Paper I): Biopsies were collected at endoscopic exam, at the time of mounting (-40 min), at the start of experiment, i.e. after a 40min equilibration time, and after 30, 60, 90 and 120 min in Ussing chamber. Biopsies were fixed in paraformaldehyde, embedded in paraffin, sectioned and stained with haematoxylin/eosin and reviewed histopathologically with time points blinded to the examiner. The specimens consisted of the epithelial cell layer, the lamina propria, the muscularis mucosae and a small part of submucosal layer. The most pronounced damage found in the epithelial layer in each specimen denoted the severity score, as follows: normal appearance score =0; depletion of goblet cells =1, small areas with lifting of epithelial cells =2; larger areas with lifting of epithelial cells = 3. Specimens were also examined for extent of epithelial damage and mucosal oedema. The extent of epithelial damage was assessed as; 0, none; 1, changes in less than 1/3 of the specimen; 2, changes in 1/3-2/3 of the specimen; and 3, changes in more than 2/3 of the specimen. The criteria for oedema were increased distance between glands and cells in the lamina propria and separation between collagen bundles. Oedema was assessed as: none = 0; minor =1; pronounced oedema = 2. An epithelial damage score was set as the sum of the variables described with a maximal value of 8 points.

Routine pathology review (paper I-IV.) Routine clinical histology assessments were conducted in all subjects. Briefly, biopsies were taken during endoscopy at the level of sigmoid colon and then fixed in paraformaldehyde. The biopsies were processed and reviewed according to local hospital routines. Colonic biopsies were normal in all controls and in patients (paper I) For UC patients, see paper IV, table 1

Light microscopy in immunohistochemistry (Paper III-IV). Biopsies from healthy volunteers (paper III and IV) and from UC patients (paper IV) were collected at endoscopy. Samples were processed for visualisation of immunostaining as described below. The light microscopic analysis was performed in a Nikon Eclipse E800 light microscope equipped with a VFM EPI-fluorescens attachment (Vector Laboratories Inc.).

Confocal laser scanning microscopy. In Paper II, confocal microscopy was used to study CRH receptor R1 and R2 in relation to mast cells. Further, the in situ hybridisations of CRH receptors in HMC-1 were observed. Tissues were prepared for confocal microscopy as described in paper II.
Transmission electron microscopy

**Preparation.** Biopsies were fixed in 2% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4) for 2 hours at room temperature, washed in several steps, incubated with 5 mg of 3,3’-diaminobenzadine tetrahydrochloride in 10 mL of 0.05 mol/L Tris buffer and 0.01% H₂O₂ (pH 7.6 at 22°C). HRP product identification was modified from Graham and Karnovsky 211 as previously described. 212 The biopsies were then washed in sodium cacodylate buffer, dehydrated and embedded in Epon (TAAB 812, Analytical standards, Gothenburg, Sweden). Ultrathin sections were cut and stained with lead citrate and examined for HRP passage routes and signs of cell damage in a TEM (JEM-1230, JEOL Sollentuna, Sweden) at 100kV or micrographs were captured in computer photo files and reviewed.

**Cell damage (paper I-III).** The route of transepithelial transport of HRP was examined by transmission electron microscopy (TEM). In the first study (paper I) the biopsies were fixed in the Ussing chamber at different time points in order to determine cell damage development over time. We also wanted to assess HRP passage over time.

**Quantification of transcellular HRP uptake (Paper II and III).** Tissues were fixed after 30 minutes in the Ussing chamber. Quantitative analysis of HRP uptake in intracellular endosomes was performed on coded high magnification photomicrographs from the surface epithelium. HRP-containing endosomes within a fixed size observation window (300 μm²) in the apical region of the colonocytes were counted and measured using a computerised image analysis system (Kontron Mop Videoplan, Kontron, Eching, Germany). Twenty photomicrographs from each treatment were examined in biopsies from three volunteers. The area of HRP-containing endosomes was calculated and averaged for each volunteer before determining group means.

**Mast cell activation (Paper II).** Mast cells were evaluated for signs of activation by image analysis. Patterns of mast cell activation/degranulation were assessed as: piecemeal degranulation, defined as the loss of granule density without granule-cell membrane fusion,184 or anaphylactic degranulation, defined as solubilisation of granular contents with fusion of inter-granular and granule-cell membranes. 123 Sections from each tissue were randomly selected, 30 mast cells were identified on each grid, analysed for loss of density and perigranular vacuolisation, and grouped into activated and non-activated.
**Immunohistochemistry in colonic biopsies**

Immunohistochemistry was used in paper II-IV to detect receptors, neurotransmitters, subepithelial inflammatory cells and nerve structures. 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 control sections were added secondary, but not primary antibody. We used different preparation and observation techniques in paper II compared to paper III and IV because of different routines in between the Linköping group and our collaborators at McMaster University where the immunohistochemistry in the second paper was conducted. The main difference in preparation and microscopy were as follows. In paper II the biopsies were snap frozen in liquid nitrogen. Cryosections were prepared and fixed with cold acetone. In paper III and IV, biopsies were immediately immersed in 4% paraformaldehyde and in PBS for 2 hours and then cryoprotected overnight in 30% sucrose before sectioned at 4-10 μm in a cryostat. In papers II-IV sections were incubated with different primary antibody layers see table 2, for specification, and followed by incubation with secondary antisera conjugated to fluorescent markers. The immunohistochemical procedure was performed according to figure 9.

![Immunohistochemistry diagram](image)

**Figure 9.** The immunohistochemical procedure used to identify expressions of neuropeptides and their receptors in human colon and in HMC-1. A light (paper II-IV) and a confocal microscope (paper II) were used for review of stained tissue.

**Molecular techniques in the HMC-1 cell line**

All molecular techniques in the, human mast cell line, HMC-1 were done by our collaborators at McMaster University, Hamilton, Canada. For detailed information on the methodology, see paper II. Briefly, we studied CRH receptor subtype expression in DNA, mRNA, and protein in the HMC-1 cell line with different molecular methods. To explore if the human mast cells express CRH receptor we used the HMC-1 cell line, which was a gift from Dr J.H. Butterfield, Mayo Clinic Rochester, USA.
subjects and methodology

<table>
<thead>
<tr>
<th>Detection of antigen</th>
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<td>Goat anti CRH (C- or N-terminal)</td>
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<td>Rabbit anti protein gene product 9.5 (PGP 9.5)</td>
<td>Biogenesis Ltd, UK</td>
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</tr>
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</table>

Table 2. Antibodies used in the studies. The secondary antisera were conjugated to fluorescent markers. In paper II FITC and Cy-5 were used as fluorescence. In papers III, IV fluorescent secondary antibodies were used, see figure 9.

RT-PCR. First we wanted to establish that the HMC-1 cells express the DNA code for CRH receptors. PCR reactions were carried out using Taq DNA polymerase with 200 ng cDNA for each amplification. Primers of the subtypes of CRH receptor R1 and R2 were confirmed by executing the software Blast. CRH-R1 primer (NM_004382, 267 bp): sense, 5'-TCTCAGGACTGCACTGCAAC-3' and antisense, 5'-AGTGCCCCAGGATGGATGC-3'; CRH-R2 primer (NM_001883, 240bp). Sense, 5'-TTCCAGGTTCTCGTGTC-3'; anti-sense, 5'-GAAGAAGAGTGGAGGGAC-3'. human glyceraldehyde-3-phosphate dehydrogenase (GADPH) primer (NM_002046, 238 bp). Sense, 5'-GAGTCAACGGATTTGCTGAC-3'; anti-sense, 5'-TGATTTGAGGGATCTCG-3'. Electrophoresis was subsequently done and visualized by ethidium bromide, using a 1-kb DNA ladder to estimate the band sizes. As a negative control for all the reactions, distilled water was used in place of the cDNA.

In situ hybridization for CRH receptor mRNA detection. Oligo-probes were synthesized by Mobix Biolab (McMaster University, Hamilton, ON, Canada) with fluorescent FITC tags, with sense strands of the oligo sequences as controls. Probes for CRH R1, R2 and control were: Oligo-1 (CRF-R2, antisense): 5'-fluoro-TCCGCGGCCTGTGTTGACTTGACCCCTGTTGAAATAC TGGGGCACG-3'). Oligo-2 (CRF-R2, sense): 5'-fluoro-CGGTCC CCGAGTAC TTCAACGGCGTCAAGTGACACACGACCAGGA-3') and Oligo-3 (CRF-R1,
antisense); (5'-fluo-CCACTAGCTGCCCCGAGGCGGCTGCAGCGGCCAGCG
GTGCC-3'). For in situ hybridization HMC-1 cells were fixed and treated with
the hybridization solutions outline in paper 2. The HMC-1 cells were reviewed
by confocal microscopy.

**Western blot.** To detect protein expression of CRH receptor subtypes R1 and R2
the HMC-1 cells were lysed and proteins quantified spectrophotometrically
using Bio-Rad DC Protein Assay kit (CA, USA). After cellular protein fraction
membranes were sequentially incubated with primary antibody to CRH receptor
1 or CRH receptor 2 (goat anti-human CRH receptor 1 or 2). HRP-conjugated
rabbit anti-goat secondary antibody (the two receptors were detected separately).
Detection of chemiluminescence was obtained by using SuperSignal West Pico
Chemiluminescent Substrate (Pierce, Rockford, IL). Ovalbumin was used as
negative size matched control.

**Immunocytochemistry.** In order to detect CRH receptors on the cell surface
HMC-1 cells were fixed in 4% formaldehyde and processed, as described earlier,
for CRH receptor R1 or R2 detection. The HMC-1 cell nuclei were stained with
propidium iodide. Secondary FITC-conjugated antibody was used. The HMC-1
cells were observed by confocal microscopy. Negative controls omitted the
primary antibodies or were incubated with a non-specific isotype IgG.

**Flow cytometry.** The HMC-1 cells were processed as described above. After
staining, the positively stained cells were counted with a FACScan. At least
10,000 cells were analyzed for each sample using the CellQuest and WinMDI.

**Methodological considerations III**

We have in paper II-IV used immunohistochemistry to show expression of
muscarinic and CRH receptors on colonic epithelial cells, eosinophilic
leucocytes and subepithelial mast cells. To exclude false positive
immunostaining, negative controls by exclusion of the primary antibody have
been used. In the second paper we show that human mast cells express CRH
receptors by several molecular methods, from detecting DNA expression to test
the functions of the CRH receptors and mast cell. This provides strong evidence
for the proposed function. In paper III and IV, we present weaker evidence
where the muscarinic receptors only are detected and tested with one molecular
method and by modulation of agonist/antagonist. Moreover the used muscarinic
antagonists are rather unspecific and do not separate different muscarinic
receptor subtypes. Further, we propose in paper IV that eosinophils produce and
release CRH upon muscarinic receptor stimulation. To strengthen the results,
eosinophilic cell culture should be used.
Statistics

In paper I data were presented as mean ± SEM or median and 25th-75th percentile. Distribution of data was tested for normality with the Shapiro-Wilks W test. In testing PD and comparison of leaky and non-leaky biopsies all biopsies were considered as individual observations. In comparisons between different intervention groups the individual observation was the calculated mean of 2-3 biopsies from each volunteer. Comparisons between two groups were done with Student’s t-test or the Mann-Whitney U-test, and for multiple group comparisons the two-way ANOVA was used. In paper II, III, and IV data were presented as mean ± SEM. In Ussing chamber experiments, the n value represents the number of volunteers or UC patients, with the mean value calculated from 2-3 biopsies for each treatment in each volunteer. Comparisons between treatment groups were done with two-way ANOVA with post-hoc tests, and Student’s t-test as applicable, and comparisons of paired data over time were assessed with the paired t-test. In comparisons between groups in HRP endosomal area (paper II and III) the total HRP filled endosomal area were calculated out of 20 views in each of 3 individuals for each treatment group. Comparisons of mast cell activation by CRH exposure was conducted (paper III) in 30 mast cells from each of two biopsies from one volunteer. Three volunteers in each group were compared. Further, (paper IV) eosinophils were counted in three subjects in each group. Comparisons were performed using Student’s t-test. In all statistical calculations, a p value less than 0.05 was considered as a significant difference. Throughout the study, StatView software (Abacus Concepts Inc, Berkley, CA, USA) was used for statistical calculations.

Statistical considerations: In the first study we used non parametric methods for the permeability calculations because of permeability results in previous studies in humans in vivo typically not being normally distributed. However, when the basis of studies grew larger it became clear that in human biopsies there was a normal distribution of the flux of both permeability markers (see figure 10 below), as well as of the PD measurements (figure 11, page 42). Moreover, when using the mean result of each volunteer/patient (instead of individual biopsies) for comparisons, the n values were low, making the non-parametric tests too blunt to detect biologically important differences.

![Figure 10](https://example.com/f10.png)

**Figure 10.** Histogram. Left: HRP flux 30-90 min (pmol/cm²/h). Right: $^{51}$Cr-EDTA papp 30-90 min. ($10^6$ cm/s). Normal distribution was confirmed by the Shapiro-Wilk W-test.
5. RESULTS

**Paper I**

In the first study our aim was to evaluate the use of endoscopic biopsies in Ussing chambers as a tool for studies of permeability to macromolecules with antigenic potential. Biopsies from the sigmoid colon of 24 healthy volunteers were mounted in Ussing chambers. $^{51}$Cr-EDTA and HRP were used as permeability markers. Mucosal permeability, with and without modulation of intestinal barrier function, electrophysiology, mucosal morphology and energy contents of the biopsies were studied over time.

The PD of the 168 studied endoscopically taken colonic biopsies was normal distributed. We found that the biopsies with a lesser negative PD than 1 SD, showed an increased permeability to $^{51}$Cr-EDTA and a drop in Isc during the 30-90 minute time period as a sign of leakage, figure 11.

![Figure 11](image1)

Figure 11. Left: Frequency histogram showing a normal distribution of PD at start of experiments. The mean value of PD was $-2.1 (\pm 1.6) \text{ mV}$ (SD). Right: Permeability to $^{51}$Cr-EDTA in biopsies grouped by PD at start. Data are presented as box plot depicting median (central line) and 25th-75th percentile; bars denote 90th percentile.

To evaluate the ability of the technique to detect ion flux and permeability changes, the mucosa was modulated with sodium caprate (C10), a medium-chain fatty acid, known to affect TJ. Exposure to C10 induced a rapid decrease in short-circuit current and a slower reversible decrease in TER. After an initial small rise, Isc fell to 47% of control during the first 30min. No difference was seen between the C10 group and the group with a washout of C10 after 10 minutes. TER decreased to 84% of control when treated with C10 and was reversed after washout, figure 13. C10 increased Papp for $^{51}$Cr-EDTA. This C10 effect was totally abolished by a washout after 10 min. Further, HRP flux was
increased in C10-exposed tissue. The C10 effect was not reversed by washout, figure 14.

The level of PD in the Ussing chamber at experimental start could discriminate between functional and non functional biopsies. Steady-state permeability with low variability was seen for $^{51}$Cr-EDTA and HRP markers during the 30-90 min period as well as an increased permeability to $^{51}$Cr-EDTA and HRP. In a subsequent experimental setup, effects of C10 were compared in conventional Ussing chamber (63.6mm$^2$) and biopsy chamber (1.76mm$^2$). Papp for $^{51}$CrEDTA and flux of HRP did not differ between resected specimens and biopsies from the same patients, figure 8. We also found that the mucosal biopsies were viable for 160 min with only a minimal increase in lactate levels during the equilibration period (biopsy to 0 minutes) but lactate levels remained stable during the experimental period. ATP levels showed a slow decline with time, but there were no significant changes from biopsy to experimental start or during the experimental period, figure 12. Only minor changes in morphology were seen in the Ussing chamber.

Figure 12. ATP and lactate levels from biopsy to mounting and during experimental period. There was an increase in lactate levels during the equilibration period, p=0.009; ATP levels showed a slow decline with time. Data are presented as mean ± SEM; n=14-19 biopsies from 10 different subjects.

Figure 13. Effects of sodium caprate (C10) on electrophysiology during the first 30 minutes. a) Short-circuit current ($I_{sc}$). b) Transepithelial resistance (TER). Values are presented as mean ± SEM of percent of control experiments in the same subjects; n=9-12 biopsies from 6 different subjects in each group; *p<0.05.
In conclusion, in paper I we show that endoscopic biopsies in Ussing chambers are reliable tools for studies of macromolecular permeability in the human colon. The mucosa was viable for up to 160 minutes in Ussing chambers. Moreover, barrier dysfunction induced by the TJ modulator, C10, was detected by alterations in electrophysiology and permeability. The technique holds great potential for clinically oriented experimental research.

Paper II

In the second study the aim was to examine the peripheral effects of CRH on mucosal barrier function in the human colon. Endoscopic biopsies were exposed to CRH in Ussing chambers and macromolecular permeability was assessed. Further, the mechanisms involved in the CRH-induced hyper-permeability were studied. In unravelling the CRH receptor expression on mast cells, the HMC-1 cell line was used.

In human colonic mucosa, CRH activated subepithelial mast cells and increased transcellular uptake of protein antigens, figure 15 and 16. Mucosal permeability to HRP was increased by CRH (2.8 ±0.5 pmol/cm²/h) compared to vehicle exposure (1.5 ±0.4 pmol/cm²/h), p=0.032, whereas permeability to $^{51}$Cr-EDTA and TER were unchanged. Electron microscopy confirmed transcellular uptake of HRP through colonocytes, figure 16.

The increased permeability to HRP was abolished by the non-specific CRH receptor antagonist α-helical CRH (9-41) (1.3±0.6 pmol/cm²/h) and the mast cell stabilizer lodoxamide (1.6±0.6 pmol/cm²/h) whereas the specific CRH receptor 1 antagonist, astressin (2.0 ± 0.15 pmol/cm²/h), and the CRH receptor 2 antagonist, antalarmin (1.8 ± 0.4 pmol/cm²/h), resulted in partial inhibition, but were not different from control (1.5 ± 0.4 pmol/cm²/h), figure 17.
In conclusion, in paper I we show that endoscopic biopsies in Ussing chambers are reliable tools for studies of macromolecular permeability in the human colon. The mucosa was viable for up to 160 minutes in Ussing chambers. Moreover, barrier dysfunction induced by the TJ modulator, C10, was detected by alterations in electrophysiology and permeability. The technique holds great potential for clinically oriented experimental research.

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In the second study the aim was to examine the peripheral effects of CRH on mucosal barrier function in the human colon. Endoscopic biopsies were exposed to CRH in Ussing chambers and macromolecular permeability was assessed. Further, the mechanisms involved in the CRH-induced hyper-permeability were studied. In unravelling the CRH receptor expression on mast cells, the HMC-1 cell line was used.

In human colonic mucosa, CRH activated subepithelial mast cells and increased transcellular uptake of protein antigens, figure 15 and 16. Mucosal permeability to HRP was increased by CRH (2.8 ±0.5 pmol/cm²/h) compared to vehicle exposure (1.5 ±0.4 pmol/cm²/h), p=0.032, whereas permeability to 51Cr-EDTA and TER were unchanged. Electron microscopy confirmed transcellular uptake of HRP through colonocytes, figure 16.

Figure 17. CRH-induced permeability was blocked by CRH receptor antagonists and by the mast cell stabilizer, lodoxamide. *) CRH vs. vehicle, p=0.032 and #) CRH vs. α-helical CRH (9-41), p=0.022. Vehicle vs. lodoxamide, astressin and antalarmin; p=n.s. Data presented as mean ± SEM pmol/cm²/h.
In human colon specimens CRH R1 as well as R2 receptors were detected by immunohistochemistry. In all sections, expression was localised in lamina propria cells close to the epithelial layer. With double staining for mast cell tryptase and CRH-R1 or CRH-R2, these receptors were found to be expressed exclusively, but not on all, mast cells. Moreover, in serial sections, mast cells were noted to express both of the CRH receptors, figure 18. To determine if human mast cells express CRH receptors, mRNA and protein expression of the two receptor subtypes CRH-R1 and R2 were assessed in the human mast cell line, HMC-1. RT-PCR and in situ hybridisation demonstrated that mRNA for both receptor subtypes was expressed in HMC-1, figure 19. These findings were confirmed at the protein level by western blot.

Figure 18. Co-localization of CRH receptor subtypes R1 and R2 and mast cells in human colonic biopsies by immunohistochemistry and confocal microscopy. Ai) Immunostaining for CRH-R1 and Aii) CRH-R2 proteins respectively are shown in blue. Bi and Bii) Costaining of both mast cells and CRH receptors, are shown as blue-grey staining.

Figure 19. CRH receptor subtypes R1 and R2 in HMC-1 cells. In immunofluorescence and confocal microscopy the CRH-R1 (a) and CRH-R2 (b) receptor protein (green) was detected and localized at the cell surface. Nuclei stained red.
In addition, immunocytochemistry demonstrated cell membrane expression of the receptors in HMC-1 cells. Moreover, protein expression was quantified by FACSscan analysis, detecting both CRH-R1 and CRH-R2 in approximately 40% of HMC-1 cells.

In conclusion, in paper II, we found that CRH activated subepithelial mast cells and increased transcellular uptake of protein antigens in human colonic mucosa. CRH receptors R1 and R2 were expressed on mast cells and CRH-receptor antagonists inhibited the permeability increase. Our findings suggest that CRH, acting via mast cells, is involved in regulating macromolecular permeability in the normal human colon.

**Paper III**

The aim of the third study was to examine the role of cholinergic signalling and mast cells in the regulation of human colonic mucosal permeability and ion secretion. Biopsies from healthy human subjects were tested with the stable Ach analogue, carbachol (Cch). We show that Cch challenge induced an increase in $I_{sc}(6\text{ min.})$ and augmented permeation of HRP compared to vehicle exposure. Further, the HRP transport showed a concentration-response pattern, with significantly increased flux compared to controls at a Cch concentration of $10^{-5}$ M, figure 20. This augmented permeation of HRP were abolished by the non-specific muscarinic receptor antagonist, atropine, but not by the nicotinic receptor antagonist, hexamethonium (Hex). This suggested muscarinic receptor mediated effects of HRP uptake. HRP uptake was also abolished by the M3 receptor antagonist, 4-DAMP suggesting that cholinergic modulation of mucosal HRP uptake involves the M3 receptor. Pre-treatment with lodoxamide showed an inhibition of HRP permeation, suggesting mast cell involvement in transcellular transport of macromolecules. Pre-treatment with the axonal fast Na$^+$ channel blocker, TTX, did not reduce the augmented HRP uptake caused by Cch. On the other hand another Na$^+$ channel blocker, lidocaine, showed abolished HRP uptake. Moreover, the increased $I_{sc}(6\text{ min.})$ and HRP uptake was abolished by the mast cell stabilizer, lodoxamide. No effects on $^{51}$Cr-EDTA or TER were noted, figure 21.

**Figure 20.** There was a dose-dependent increased permeation of HRP when the serosal side of biopsies was exposed to Cch.
the axonal fast Na⁺ channel blocker, TTX. The increased permeation was abolished by muscarinic receptor antagonists. The present findings suggest that acetylcholine is involved in ion secretion as a result of muscarinic receptor agonism. Further, Cch increased the ion secretion as a result of muscarinic receptor agonism. The muscarinic receptor M1 (red) was only found in epithelial cells of the colonic crypts. In contrast, the M2 and M3 receptor subtypes were detected in submucosal immune cells. Mast cells (green) did not express M2 receptors. Similarly, muscarinic receptor M3 (red) and mast cells (green) were not co-localized. Nerve interaction with submucosal mast cells. Mast cell tryptase is stained with green and nerve PGP is stained with red. Bar indicates 50μm.

**Table:**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Flux HRP (30-90 min) (pmol/μm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VE</td>
<td>1.0</td>
</tr>
<tr>
<td>Ca</td>
<td>2.0</td>
</tr>
<tr>
<td>Atr+C</td>
<td>3.0</td>
</tr>
<tr>
<td>DAMP+C</td>
<td>4.0</td>
</tr>
<tr>
<td>M3</td>
<td>*</td>
</tr>
<tr>
<td>M1</td>
<td>#</td>
</tr>
<tr>
<td>M2</td>
<td>#</td>
</tr>
<tr>
<td>M1+Cch</td>
<td>*</td>
</tr>
<tr>
<td>M3+Cch</td>
<td>#</td>
</tr>
<tr>
<td>TTX+C</td>
<td>#</td>
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</tbody>
</table>

**Figure 21.** Augmented permeation of HRP was abolished by the muscarinic receptor antagonists atropine and 4-DAMP, but not by the nicotinic receptor antagonist hexamethonium. HRP permeation was further inhibited by the mast cell stabilizer lodoxamide and the Na⁺ channel blocker, lidocaine, but not with the axonal fast Na⁺ channel blocker, TTX.

**Figure 22.** Muscarinic receptor expression in human colonic mucosa. a) The muscarinic receptor M1 (red) was only found in epithelial cells in the crypt regions. Mast cells (green) were often located close to M1 positive cells. b) Muscarinic receptor M2 (red) was expressed in submucosal immune cells. Mast cells (green) did not express M2 receptors. c) Similarly, muscarinic receptor M3 (red) and mast cells (green) were not co-localized. d) Nerve interaction with submucosal mast cells. Mast cell tryptase is stained with green and nerve PGP is stained with red. Bar indicates 50μm.
Results

Immunohistochemistry showed staining for all the tested subtypes of muscarinic receptors. The cholinergic M1 receptor was only detected in epithelial cells of the colonic crypts. In contrast, the M2 and M3 receptor subtypes were detected in subepithelial cells, presumably of inflammatory origin, but not in mast cells. Further, subepithelial mast cells were detected in close contact with enteric nerve filaments, figure 22.

In conclusion paper III shows the effects of cholinergic mediators on macromolecular permeability and electrophysiology in human colonic mucosa. The increased permeation was abolished by muscarinic receptor antagonists. Further, Cch increased the ion secretion as a result of muscarinic receptor agonism. The present findings suggest that acetylcholine is involved in regulating permeability to protein antigens and ion secretion in the normal human colon.

Paper IV

In the fourth study our aim was to elucidate macromolecular permeability and ion secretion in macroscopically non-inflamed colon in UC. Further, we wanted to characterize the mechanism involved. In this study we present a novel explanation for increased macromolecular uptake in patients with UC.

Permeability to HRP was increased in patients with UC in remission compared to controls. Flux of HRP was 2.3 times higher in UC patients than controls but there was no difference in $^{51}$Cr-EDTA permeation. Both Isc and TER at start was higher in the UC group compared to controls suggesting increased ion flux in UC patients.

UC biopsies, with an increased permeability to HRP showed normalized HRP permeation compared to controls, by pre-treatment with the muscarinic receptor antagonist Atr, the CRH antagonist $\alpha$-helical CRH (9-41) and the mast cell stabilizer lodoxamide tromethamine, respectively. Immunofluorescence showed increased numbers of CRH expressing eosinophils in the UC group. Further the muscarinic receptors, M2 and M3, were detected on the subepithelial eosinophilic leucocytes.

In conclusion, we present, in paper IV, evidence of a signalling cascade involving cholinergic receptors on CRH-producing eosinophils and activation of mast cells, leading to increased macromolecular uptake across the colonic mucosa, which may be involved in the inflammatory process in UC.
Studies of intestinal permeability in humans have previously mainly been carried out with in vivo techniques. Such studies are easy to perform but give limited information on mechanisms involved in epithelial barrier function. The Ussing chamber technique for in vitro studies of epithelial function is well established for studies of intestinal ion transport, drug absorption, and permeability to various-sized marker molecules and antigens in gut mucosa of laboratory animals. In vitro studies of barrier function in human intestine have so far essentially been limited to specimens from patients undergoing GI-surgery. These studies have some obvious drawbacks since surgical patients have possible alterations in physiology due to medication, surgical stress, malnutrition and other factors which influence the mucosal barrier function. Furthermore it is difficult to find adequate control groups as, by definition, patients in need of bowel surgery are not healthy. The use of endoscopic biopsies of human intestinal mucosa in Ussing chambers provides an opportunity for detailed studies of epithelial function in healthy individuals, as well as in patients with various diseases. A few groups have used endoscopic biopsies of human intestine to assess ion secretion but only one previous study has been conducted to determine mucosal permeability to protein antigens. In the initial study the objective was to develop and validate the endoscopic biopsy technique for Ussing chamber studies of macromolecular permeability in human intestine. We showed that the mucosa was viable for up to 160 minutes with only minor changes in morphology and stable levels of ATP and lactate. This suggests that viability characteristics for colonic biopsies in Ussing chambers were better than for surgical specimens of human ileum in our previous study. Our data demonstrate that biopsies with a PD less negative than –0.5 mV at start should be excluded since the biopsies were leaky and less stable in their electrophysiological variables, suggesting impaired cellular function, disruption in barrier and possibly edge leakage. In order to test modulation of the barrier function of the biopsy, C10 was used. From earlier studies it is known that C10 acts on TJ by reducing ATP formation in the mitochondria and increasing the TJ dependent paracellular permeability. Exposure of biopsies to C10 confirmed the expected effects with increased TJ permeability to 51Cr-EDTA and mitochondrial changes seen by transmission electron microscope. Sodium caprate exposure also increased trans-cellular uptake of the protein antigen HRP. We have shown the principal methods to study electrophysical parameters and permeability changes in a small area of exposed human colonic mucosa and the importance to evaluate the PD as a discriminator of viability for the studied part of the intestine. Our findings show that endoscopic biopsies in Ussing chambers are reliable tools for studies of macromolecular permeability in the human colon. The use of biopsies in Ussing chamber permeability studies allow follow up of new treatments in patients with various diseases in the gastrointestinal tract. For example, medications for IBD and IBS with poorly described effect on the intestinal barrier function. The use of biopsies in Ussing chamber is not only limited to the colon but can be used in...
CRH has been shown to be one of the most important mediators of stress-induced abnormalities in mucosal function in animal models.\textsuperscript{123,187,189} Reports have also suggested involvement of subepithelial mast cells in regulation of transcytosis of antigens.\textsuperscript{181,217} In addition, mast cells seem to be activated by CRH, resulting in increased HRP flux,\textsuperscript{123} mucin release and increased ion secretion.\textsuperscript{185} In paper II we wanted to elucidate the link between CRH, mast cells and increased transcellular permeability in the human intestine. We were able to demonstrate functionality of the CRH receptors in the colonic biopsies, with a clear dose-response pattern to CRH exposure, and abolished permeability effects when a non-selective CRH receptor blocker was added. Further, transmission electron microscopy revealed that CRH activated subepithelial mast cells, which was corroborated by the inhibiting effects of pre-treatment with the mast cell stabiliser lodoxamide. Moreover, we clearly demonstrated CRH receptor expression on the mast cells in the HMC-1 line. We found CRH receptor mRNA and expression of the receptor subtypes on the cell membranes. In human colonic mucosa, we also found CRH receptor expression exclusively on mast cells in the lamina propria, although not expressed on all mast cells. Our findings suggest that CRH receptors are involved in the regulation of the mucosal barrier, emphasizing the need to further elucidate the CRH – CRH receptor – mast cell – epithelial cell cross-talk. However from animal studies it is clear that also other putative stress modulators than CRH are involved in regulation of the gut barrier permeability.

In the third paper we wanted to investigate a possible link between the cholinergic signalling and mast cells in the regulation of human colonic mucosal functions. Gareau et al.\textsuperscript{124} recently showed increased colonic uptake of HRP as an effect of increased cholinergic nerve activity due to maternal separation stress in rat pups. Moreover, treatment with the Ach antagonist, Atr, prevented stress-induced increase of HRP uptake in rat jejunum.\textsuperscript{152,186} Increased uptake of HRP via endosomes and increased ion secretion in rodents was shown due to activation of the muscarinic receptor 3 (M3) by the Ach analogue, Cch.\textsuperscript{122} These results were also supported by studies in animals showing increase in transcellular transport of large protein antigens by cholinergic challenge.\textsuperscript{122,218} To further clarify the role of cholinergic signalling in mucosal permeability we used biopsies from healthy volunteers. We showed a clear dose-response to Cch in uptake of HRP supporting the notion that signalling for transcellular HRP permeability is receptor mediated. Additionally, immunohistochemistry showed muscarinic receptor subtypes in colonic epithelium (M1 subtype) but also on subepithelial cells which in the fourth study proved to be eosinophils expressing the M2 and M3 receptor subtypes. Immunostaining did not show any co-localisation of muscarinic receptors and mast cells. Taken together, Ach acted on, presumably, eosinophils which in turn activated mast cells to increase the...
transcellular transport of HRP. The increase in Isc could be explained as a direct effect on muscarinic receptor M1 expressing colonocytes in the epithelium.

It is known that persistent stress and stressful life events seem to increase the risk for exacerbations of UC. It is also known that increased muscarinic neural representation and enhanced expression of CRH in mucosal inflammatory cells and in mucosal macrophages. However, although immunohistochemistry was used, the CRH producing cells were not well defined in previous reports. In the fourth paper we used colonic biopsies from patients with UC in remission and healthy volunteers as controls. Initially, in Ussing chamber experiments we found a more than doubled permeability to the protein antigen HRP in the UC group compared to healthy controls. This is well in line with previous studies. Interestingly the increased HRP permeability could be normalised by treatment with a CRH receptor antagonist, muscarinic receptor antagonist and mast cell stabiliser. We also perceived that baseline Isc was higher in UC patients compared to controls which may be explained by an increase in levels of TNF-α and subsequent modulation of mechanisms involving intracellular increase of Ca²⁺ as well as by metabolites of the eicosanoids, which are elevated in UC. In order to scrutinise the immunohistochemical appearance in the colonic biopsies we found co-localisation of the cholinergic receptor M2 and M3 subtypes on eosinophils, which has earlier not been described in the literature. In UC biopsies there was a fivefold increase of eosinophils compared to healthy controls. Eosinophils have been reported to localize close to cholinergic neurons and mediate nerve remodelling and induce release of Ach from cholinergic nerve cells. Additional immuno-histochemical staining revealed eosinophils as the source of CRH in colonic mucosa. Moreover, the eosinophils were located in close proximity to mast cells, which indicates the possibility for CRH signalling between eosinophils and mast cells. Our results, however, contrast results in earlier studies and need to be validated in additional modalities for example detection of CRH by in situ hybridization.

In conclusion, in study II-IV we have demonstrated a chain of events leading to increased permeability to the protein antigen HRP in biopsies from healthy volunteers and patients with UC. The important steps are summarised in figure 23. This explanatory model may have implications for understanding of the pathogenesis of UC and future treatment of the disease. However several questions still remain to be resolved. Description of the innervation of eosinophilic leucocytes is sparse in literature. We found expression of Ach receptors on eosinophils but cholinergic signalling may also come from a “non-neuronal cholinergic system" a signalling system described for airway inflammatory diseases and recently also suggested in the colon in UC. One of the most important next steps will be to characterize the neurons innervating mast cells and eosinophils.
Fig. 23. A cholinergic signal, probably via the brain-gut axis signalling, acts on muscarinic M2 or M3 receptors on the eosinophils, which leads to activation and release of CRH. Next, CRH R1 and CRH R2 receptors on the mast cells are activated (by CRH), leading to release of factors that increase macromolecular uptake across the epithelium.

Besides CRH and Ach, a variety of signalling substances are described to affect mast cells and eosinophils. Among these are Neuropeptide Y, somatostatin, vasoactive intestinal polypeptide epinephrine, norepinephrine and Substance P (SP). SP is suggested as an important mediator in neuroimmune crosstalk in IBD. There is however conflicting evidence of NK-1 receptors on mast cells and eosinophils. Challenge with NK-1 receptor agonist and antagonist and immunohistochemistry would elucidate the role of SP in mucosal permeability and neuroimmune crosstalk in IBD. Another issue of importance is the signalling between the mast cell and the epithelium. Mast cells contain many different effector substances including histamine, tryptase, TNF-α, interleukins (1, 3, 5, 6, 8, 13, 16, 18), TGF-β, NGF, VIP and eicosanoids, several with capability to change mucosal permeability.

Based on the knowledge presented in this thesis thoughts of future treatment of stress induced intestinal diseases include medication directed to prevent...
increased permeability across the epithelium. The CRH receptor seems to be a target for mast cell modulation. Further, release of CRH from eosinophils could be inhibited by a proposed selective muscarinic receptor antagonist.

Persistent psychological stress seems to be a pre-requisite for stress induced intestinal diseases. Patients with these intestinal disorders should have information and advice on avoidance and coping with persistent daily stress in order to prevent aggravation of an ongoing process or exacerbation of quiescent disease. Perceived and physical stress during surgery in these patients may also affect the course of their intestinal disease Considerations should therefore be taken during the surgical care of the patient in order to minimize stress induced barrier dysfunction.
7. CONCLUSIONS

- Endoscopic biopsies of human colon are viable in the Ussing chamber and are reliable tools for studies of mucosal permeability to protein antigens.

- Potential difference measurements discriminates colonic biopsy viability in the Ussing chamber system.

- Transcellular uptake of HRP in human colonic mucosa is regulated by CRH acting on subepithelial mast cells.

- Human colonic subepithelial mast cells and the human mast cell line, HMC-1, express CRH receptor subtypes R1 and R2.

- Short circuit current and transcellular HRP uptake across human colonic mucosa is regulated via muscarinic receptors.

- Cholinergic signalling regulates colonic transcellular HRP uptake via subepithelial mast cells.

- Human colonic subepithelial eosinophils express CRH and the muscarinic receptor subtypes M2 and M3.

- Increased numbers of colonic subepithelial eosinophils are seen in ulcerative colitis in remission compared to healthy controls.

- Colonic permeability to HRP is increased in UC colonic biopsies and is normalized by blocking of muscarinic receptors, CRH receptors and mast cells, respectively.
8. TACK

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Kollegorna på kirurgkliniken, tack för att ni har hjälp till på många olika sätt genom åren att förverkliga denna bok. Speciellt tack till Anders för den kreativa schemaläggningen under 2007 och Pelle för att du tog akuten den där fredagen…


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Författaren lyfter på hatten och tackar
9. Svensk Sammanfattning


Avhandlingen handlar om regleringen av tjoctarmens barriär hos människa vid friska förhållanden och vid ulcerös kolit. Det första arbetet består av metodutveckling där Ussingkammaren (Hans Ussing, dansk fysiolog), modifierats och validerats för användandet av små tarmslumhinnebitar tagna vid endoskopisk tjoctarmundersökning. Tidigare användes större tarmbitar från operationspreparat då patienter opererades i tjoctarmen. Andra och tredje arbetet behandlar stresshormonerna acetylkolin och CRH påverkan på barriärfunktionen hos friska frivilliga försökspersoner. Det sista arbetet i avhandlingen handlar om hur tjoctarmens barriärfunktion hos patienter med ulcerös kolit är förändrad jämfört med friska frivilliga försökspersoner. Vidare studerades om det var någon skillnad i hur barriärfunktionen kunde påverkas med olika blockerande substanser för bl.a. acetylkolin och CRH. För att kunna svara på de frågor som vi har ställt oss har vi använt olika experimentella metoder. I Ussingkammaren bildar själva tarmslumhinnebitan en barriär mellan två halvkammare och passage av markörämne (HRP) från ena sidan, genom slumhinnebiten till den andra, kan mätas. Vi har också genomfört olika slags mikroskopiskt, antikroppsinläggningar, olika molekylbioologiska metoder och studerat en odlad mast cell linje, HMC-1.

Huvudresultaten i avhandlingen visade att modifieringen av Ussingkammat tekniken var tillförlitlig och kunde användas för fortsatta försök. Vidare så visade vi att tjocktarmen glaserade åter förändras med CRH och acetylkolin liknande ämne med ett ökat uppdrag av HRP. Dessa effekter är indirekta och beroende av flera celltyper i tarmslumhinne. De sk. mast cellerna, immunceller, intar en central roll i denna reglering. I det fjärde arbetet påvisas att ytterligare en immuncelltyp, eosinofiler, deltar i regleringen av genomsläpppligheten. Patienter med ulcerös kolit har, trots lugnt skede av sjukdomen, ett ökat uppdrag av HRP. Vid behandling av slumhinnebitarna i Ussingkammaren med hämmer av CRH och acetylkolin receptorer samt mast celler kunde den ökade genomsläpppligheten normaliseras.

Slutsatsen man kan dra från avhandlingsarbetet är att CRH och acetylkolin påverkar immunceller som sedan ökar tarmslumhinnebittens uppdrag av farliga ämnen och kan leda till inflammation i tjoctarmen. Hos patienter med ulcerös kolit är tjocktarmelumhinne bättre genomsläpplig för HRP än hos friska frivilliga försökspersoner, vilket kan vara en delförklaring till sjukdomens uppkomst.
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