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Autoantigens in Inflammatory Bowel Disease and Primary Sclerosing Cholangitis

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Abstract
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Inflammatory bowel disease (IBD) comprises diseases that are characterized by chronic or relapsing inflammation of the gastrointestinal tract. Primary sclerosing cholangitis (PSC) is an extraintestinal manifestation in IBD. Immunoreactivity against an autoantigen that is expressed both in the gastrointestinal tract and the biliary tract could be the link between these diseases. A possible source of such an antigen is goblet cells.

Immunostainings of normal human tissues using IBD patient sera showed goblet cell immunoreactivity against goblet cells in all parts of the gastrointestinal tract. The most frequent immunostaining was found against goblet cells in the appendix against which 84% (42/50) of IBD patients compared to 8% (4/50) of healthy blood donors showed immunoreactivity. To identify the corresponding antigen we used three different approaches, investigation of immunoreactivity to different candidate proteins compared to IBD sera, immunoscreening of an appendiceal cDNA library, and immunoprecipitation of protein lysates from mucin producing cells followed by SDS-PAGE and 2D gel electrophoresis. These approaches led to the identification of several candidate autoantigens of which complement C3 is the most promising.

A novel staining pattern with strong immunoreactivity to granules and the apical membrane of biliary epithelial cells was identified with 35% (12/34) of PSC sera compared to none of healthy controls (n=28). Screening of a cDNA library from normal human choledochus identified PDZ domain containing 1 (Pdzk1) and Glutathion S transferase theta 1 (GSTT1) as potential candidates. Pdzk1 is an interesting candidate which is expressed in the intestinal tract and bile ducts. GSTT1 antibodies were not specific for PSC and are thought to develop as an alloimmune response in patients with the *GSTT1*-null genotype.

In conclusion, we have identified specific immunoreactivity to goblet cells and biliary epithelial cells using sera from patients with IBD and PSC respectively. We have also identified several potential autoantigens.

Keywords: inflammatory bowel disease, primary sclerosing cholangitis, autoimmunity, autoantigen, goblet cell

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To my family

List of papers

This thesis is based on the following papers[1], which will be referred to by their roman numerals:

I **Ardesjö B**, Portela-Gomes GM, Rorsman F, Gerdin E, Lööf L, Grimelius L, Kämpe O and Ekwall O. **Immunoreactivity Against Goblet Cells in Patients with Inflammatory Bowel Disease.** *Inflamm Bowel Dis* 2008; 14:652-661.

II **Ardesjö B**, Rorsman F, Portela-Gomes GM, Grimelius L, Kämpe O and Ekwall O. **Investigation of goblet cell autoantigens in patients with inflammatory bowel disease.** *Manuscript*

III **Ardesjö B**, Hansson CM, Bruder CEG, Rorsman F, Betterle C, Dumanski JP, Kämpe O and Ekwall O. **Autoantibodies to Glutathione S-transferase theta 1 in patients with primary sclerosing cholangitis and other autoimmune diseases.** *J Autoimmun* 2008, Jan 31, doi:10.1016/j.jaut.2007.11.008

IV **Ardesjö B**, Portela-Gomes GM, Rorsman F, Grimelius L, Kämpe O and Ekwall O. **Immunoreactivity against bile duct epithelial cells and identification of PDZ domain containing 1 as a novel autoantigen in Primary sclerosing cholangitis.** *Manuscript*

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Contents

Abbreviations

AIH	autoimmune hepatitis
APC	antigen presenting cell
APC	adenomatous polyposis coli protein
BCR	B cell receptor
BECs	biliary epithelial cells
complement C3	complement component 3
CD	Crohn's disease
CFTR	cystic fibrosis transmembrane conductance regulator channel
DC	dendritic cell
GST	Glutathione S-transferase
GSTT1	Glutathione S-transferase tetha 1
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
MadCAM-1	mucosal addressin cell adhesion molecule 1
MDP	muramyl dipeptide
MDR	multi drug resistance
MHC	major histocompatibility complex
MICA	MHC class I chain-related molecule A
MUC	mucin
NFκB	nuclear factor-κB
NHE3	Na^+ H^+ Exchanger 3
NHERF-3	NHE3 regulatory factor 3
NK cell	natural killer cell
OCTN	organic cat ion transporter
PAMP	pathogen associated molecular pattern
pANNA	peripheral anti-neutrophil nuclear antigen
PBC	primary biliary cirrhosis
Pdzk1	PDZ domain containing 1
PSC	primary sclerosing cholangitis
SLE	systemic lupus erythematosus
SNP	single nucleotide polymorphism

TCR	T cell receptor
TFF	trefoil factor
Th cell	T helper cell
TLR	toll-like receptor
TM	tropomyosin
T reg	regulatory T cell
UBE4A	ubiquitination factor E4A
UC	ulcerative colitis

Introduction

General immunology

The first defence a micro-organism encounters when trying to invade the body is an epithelial barrier in the form of the skin or the epithelium of the gastrointestinal, urogenital or respiratory tract. Directly under the epithelia reside phagocytes which can engulf and digest the invading microorganisms. The phagocytes also send signals that induce inflammation and recruit other immune cells to the site. This is part of the so called innate immune system which is fast but not specific. Later on the adaptive immune system is activated by binding of receptors to specific epitopes of the microorganism, but the activation of this system also depends on signals from the innate immune system. Working together these systems provide a good defence against foreign antigens.

Innate immunity

The innate immune system consists of immunologically active cells (e.g. granulocytes, macrophages, mast cells, dendritic cells and natural killer cells) and molecules (e.g. complement factors, acute phase proteins, cytokines and defensins). The innate immune system is a rapid system that does not depend on proliferation, but on the other hand it is unspecific. The receptors in the innate immune system recognize pathogen associated molecular patterns (PAMPs) on the invader. These patterns are often specific for microbial pathogens, crucial for survival of these pathogens and shared by a whole class of pathogens. Many of the PAMP recognizing receptors are located in the membranes of the phagocytic cells and on encountering with a microorganism the binding of the receptor will trigger phagocytosis of the organism. Binding to other receptors such as Toll-like receptors (TLRs) leads to expression of co-stimulatory molecules and cytokines needed for the activation of the adaptive immune system. Other cells release toxic granules on encountering the pathogens, which kill the cells that are later phagocytised by macrophages. In contrast to the adaptive immune system the innate immune system can not recognize self structures. Hence the signals from the innate immunity needed to activate the adaptive immune system are only present when there is a foreign invader present, and this prevents the

adaptive immune system from attacking any self structures they might recognize.

Adaptive immunity

The adaptive immune system consists of lymphocytes known as B cells and T cells. This is a highly specific system, able to recognise an infinite variety of antigens through unique receptors. This system is slower on the first encounter with the invader than the innate immune system because it depends on clonal expansion of the cell with the specific receptor, however on the next encounter memory cells with the specific receptor ensures a faster response.

B cells and their development

B cells develop in the bone marrow where their unique receptors are produced in different stages.

The B cell receptor (BCR) consists of two smaller light chains and two larger heavy chains. The BCR has a Y shape where one light chain and part of one heavy chain form each arm (FAB fragments) while the lower parts of the heavy chains form the trunk (FC fragment) and are bound by sulphide bonds. The outer parts of the Y-arms are the variable regions of both types of chains while the rest of the molecule is made up of the constant regions of the chains. The tips of the arms are the binding sites of the BCR and are made up of hypervariable regions that will bind the epitope on the microorganism, also called the antigen. The two binding sites enable cross-linking. The BCR is bound to the cell surface by a transmembrane part of the FC fragment.

The gene for the B cell receptor consists of several segments for each part of the receptor and these segments are joined and rearranged in several steps during the development of the B cell. The variable region of the heavy chain is made up of three segments, the variable (V), diversity (D) and joining (J) segments which are joined by somatic recombination to produce the variable region, and a segment for the constant part (C) is also joined to these segments. Addition or subtraction of bases at the sites of joining of the different segments enhances the specificity. The heavy chains are expressed together with the surrogate light chains as the pre-B cell receptor. If the receptor is expressed successfully the rearrangement of the light chain starts with joining of the V, J and C segments. If this rearrangement leads to expression of a functional BCR, the immature B cell survives and can leave the bone marrow.

The immature B cell is transported through the bloodstream to peripheral lymphoid tissues where they undergo selection for self tolerance and subsequently for their ability to survive. The cells that pass these selections undergo further development and these naïve B cells then re-circulate through the peripheral lymphoid tissues to encounter their appropriate antigen and

become activated. When B cells have encountered their antigen, they receive a signal through the BCR but they also need signals from cytokines provided by CD4 positive T cells, called T helper type 2 (Th2) cells, to become activated. When the B cells get these signals, they proliferate and undergo somatic hypermutation during which point-mutations occur at a high rate in the V-region of the receptor-gene generating mutant receptors that are expressed. The cells expressing receptors with improved specificity survive and undergo clonal expansion which renders a clone of B cells with the same receptor specificity. Most of these B cells develop into plasma cells which are effector cells acting through the production of antibodies. Some cells develop into memory B cells which can recognize the antigen and lead to a faster immune response on a second encounter.

Antibodies

The BCR can also be produced with different constant regions lacking the transmembrane part. This receptor is instead secreted from the B cells and is called antibody. Several different forms of antibodies with different constant regions exist and binding of the antigen by these different antibodies lead to different actions. The antibodies are also called immunoglobulins, in short Ig. The different types are named after the constant region segment (α,γ,μ and ε) and are called IgA, IgG, IgM and IgE. Antibodies can bind soluble antigens and fight the pathogens in three different ways. First, the binding of antibodies to the antigen can prevent the antigen from binding to the epithelium and thereby stop it from establishing an infection, this is called neutralization. Second, an antibody bound to an antigen can be recognized by a phagocytic cell that binds to the Fc region of the antibody and engulfs the antigen attached to the antibody; this is called opsonisation. Third, antibodies bound to their antigen can also start the complement cascade which leads to recruitment of immune cells and direct action of the complement system creating a pore in the cell membrane of the pathogen leading to destruction of the pathogen.

T cells and their development

T cells develop in the thymus (hence the T). They also have unique T cell receptors (TCRs).

T cells recognize intracellular pathogens or antigens that have been engulfed. Short parts of peptides from these pathogens are presented on antigen presenting cells (APCs) in complex with major histocompatibility complex (MHC) I and II and are bound by the TCRs. MHC I is expressed on all nucleated cells and presents antigens degraded in the cytosol. MHC II is expressed only on APCs and presents antigens that have been degraded in endocytic vesicles.

The TCR of most T cells consists of two chains, one α- and one β-chain. They have a structure similar to the BCRs Fab fragment, and each chain has a constant and a variable region but they also have a transmembrane part. A

minority of T cells have a TCR with a γ- and a δ-chain which also has a structure similar to the Fab fragment. The TCR is also generated through gene rearrangement as described above for the BCR. The α-chain gene has V, J and C segments and the β-chain has V, D, J, and C segments. The β-chain is rearranged first and expressed with a surrogate α-chain. If the cell can express this pre-TCR together with the CD3 signalling molecule the expression of the co-receptors CD4 and CD8 starts as well as the rearrangement of the α-chain gene.

The cells with expression of two co-receptors are called double positive thymocytes and make up the vast majority of T cells. In the thymus these double positive cells undergo so-called positive selection through interaction with self-peptides on MHC presented by dendritic cells. The cells that recognize one of the complexes survive, undergo maturation and express a high level of TCRs on their surface and at the same time they cease to express one of the co-receptors and become a CD4+ cell if they recognized an MHC II or a CD8+ T cell if they bound to an MHC I. There is also a negative selection of T cells which takes place during and after the double positive stage. The negative selection is mediated mainly by dendritic cells and medullary thymic epithelial cells which present self antigens in complex with MHC. The T cells that bind strongly to the MHC:self antigen complex receive signals which make these cells go into apoptosis. In this way the T cells with potential to react against self molecules are eliminated.

The surviving T cells leave the thymus and re-circulate to the peripheral lymphoid tissues. When these naïve T cells encounter their antigen presented by an APC together with an MHC they become activated. The T cells that recognize the MHC I:peptide complex and have the CD8 receptor are cytotoxic T cells that can kill the cell that present their antigen by releasing effector molecules. The T cells that recognize MHC II and express CD4 are called T helper cells and develop into three groups. T helper 1 (Th1) cells can activate macrophages that have been infected with intravesicular pathogens so they can degrade these pathogens. Th2 cells can activate B cells which have bound antigens to their antibodies which have been internalised and presented on MHC II. The signals from the Th2 cell induce proliferation and isotype switching in the B cell, which develops into a plasma cell. There is usually one type of Th cells present at an infection site. This is because Th1 cells produce and release cytokines that inhibit the development of Th2 cells and vice versa. The Th cell type that is activated first represses the activation of the other cell type. Recently a new type of T helper cell has been identified, the so-called Th17 cell, and cytokines from both Th1 and Th2 cells have been shown to have an inhibiting effect on these cells. Th17 cells have been implicated in both infectious and autoimmune diseases and can mediate clearance of pathogens and autoimmune cell damage (1)

There is also a subset of T cells that have regulatory functions. The most well characterized regulatory T cell is the CD4+ T cell that expresses CD25 (the α-chain of the interleukin (IL)-2 receptor). This cell binds to a self-

antigen and can release cytokines which have regulating effects on other T cells leading to inhibition of proliferation. They are also suggested to be able to induce contact dependent apoptosis in other T cells (2).

Autoimmunity

Although several mechanisms prevent the adaptive immune system from reacting to self-molecules, this reaction still occurs and causes autoimmunity.

Most lymphocytes that are reactive to self peptides are, as mentioned above, eliminated; however some are not. To prevent the activation of these lymphocytes tolerance is induced in these cells. This is due to strong continuous signalling through their TCR or BCR when they are chronically exposed to and bind to their antigen which is expressed in a constant concentration and this leads to the apoptosis of the lymphocytes. Another mechanism that prevents self-reactive T cells from becoming activated is the induction of anergy in these cells caused by the lack of stimulatory signals induced by the innate immune system. This makes these cells go into a state where they can no longer be activated and also affects the B cells that cannot get an activating signal from these autoreactive T cells. All these mechanisms are prone to error because none of them can truly distinguish self from non-self. The self-reactive lymphocytes with low affinity do not make any response to their antigen and therefore escape also the peripheral tolerance. Several autoreactive lymphocytes that are anergic or have low affinity circulate within the body at the normal physiological state without causing disease.

There are several ways in which these self-reactive lymphocytes can get activated. A change in the availability or the form of the antigen can trigger activation. An example of this is when antigens that are normally intracellular or behind a tissue barrier are released due to tissue death or inflammation (Fig. 1A). Another cause of activation of low affinity B cells is hypermutation causing high affinity to the antigen. There are also mechanisms that can activate anergic self-reactive lymphocytes as well as low affinity lymphocytes. One is molecular mimicry which occurs when a pathogenic antigen by chance or design is similar to a self-protein and the lymphocyte recognizing

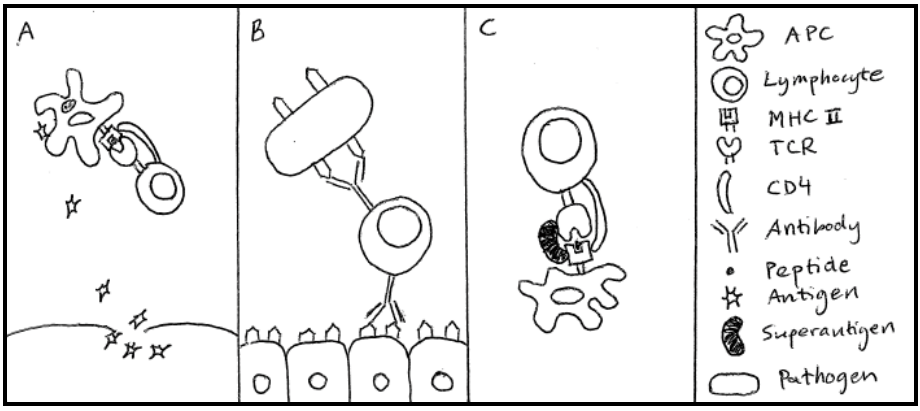

Figure 1. Three different ways of breaking self tolerance: Release of normally unavailable antigens by breakage of a tissue barrier and activation of non-tolerised cells (A), production of cross-reactive antibodies due to molecular mimicry (B) and polyclonal activation of autoreactive T cells mediated by binding of a superantigen to the MHC and TCR (C).

this antigen is activated and can then bind to self-antigen and mediate tissue damage (Fig. 1B). The lymphocytes with low affinity can be activated if they receive a strong co-stimulatory signal caused by for example infection, and a pro-inflammatory signal that is strong enough can even activate anergic lymphocytes. Another activating mechanism is caused by molecules called superantigens. These are proteins produced by pathogens that can activate CD4+ T cells by binding to both to the outer part of the MHC II molecule and the Vβ domain of the TCR (Fig. 1C). The activated self-reactive lymphocytes do not have to cause autoimmunity; they can be inhibited to proliferate by regulatory lymphocytes. The lymphocytes also have intrinsic limits to proliferation and survival which can help limiting the autoimmune response.

The result of activated self-reactive lymphocytes that escape all mechanisms of inhibition can be autoimmune disease. The classical definition of an autoimmune disease from 1957 (3) includes four criteria.

1. The existence of an autoantibody or cell mediated immunity.
2. The identification of the corresponding antigen.
3. The induction of disease in an experimental animal by immunisation with the antigen.
4. The transfer of disease to a healthy individual by transfer of T cells, B cells or autoantibodies.

Even though as much as 3% of the population is affected by an autoimmune disease (4) the mechanisms underlying these diseases are not fully understood. Genetic associations have been reported in several autoimmune diseases and familial clustering in autoimmune diseases is common. Individuals with one autoimmune disease are shown to have an increased risk of being

affected by a second autoimmune disease. Different human leukocyte antigen (HLA) alleles have been shown to be either protective or to increase the susceptibility for different autoimmune diseases (5). The autoimmune diseases can be divided into organ-specific and systemic autoimmune diseases depending on whether specific organs are attacked by the autoimmune cells or whether several tissues of the body are affected. Example of diseases in the organ-specific group is insulin-dependent diabetes mellitus and multiple sclerosis, and examples of systemic disease are rheumatoid arthritis and systemic lupus erythematosus (SLE).

Autoantigens are self structures identified through reaction with autoantibodies or self-reactive T cells that are associated with autoimmune diseases. Many B cell autoantigens have been found through immunohistochemistry, immunoblotting and screening of cDNA libraries. T cell autoantigens are more difficult to identify and verification of their association to autoimmune diseases is also complicated to achieve. The mechanism of effect in autoimmune diseases is mediated both through autoantibodies and through T cells, and has been classified in analogy with type II-IV hypersensitivity reactions (6). Type II reactions are antibody mediated with cell surface antigens resulting in phagocytosis or complement mediated lysis as in autoimmune haemolytic anaemia (7), receptor mediated stimulation as in Graves' disease (8), or receptor blockade as in myasthenia gravis (9). Type III reactions are immune complex mediated with extracellular antigens, matrix derived or soluble, and can be exemplified by SLE (10). Type IV reactions are T-cell mediated, organ specific destructive diseases and the antigens proposed are often intracellular as glutamic acid decarboxylase in insulin-dependent diabetes mellitus (11) and 21-hydroxylase in Addison's disease (12). A number of autoantibodies are of value as disease-specific diagnostic markers. The occurrence of autoantibodies often precedes the clinical onset of the disease (13), and can thus be used to screen persons at risk of developing the disease. The titres of some antibodies are also correlated to disease activity, e.g. anti-dsDNA antibodies in SLE (14), while others are of less value for monitoring disease.

Inflammatory bowel disease

Inflammatory bowel disease (IBD) comprises diseases that are characterized by chronic or relapsing inflammation in the gastrointestinal tract. The two major forms of IBD are Crohn's disease (CD) and ulcerative colitis (UC). These two diseases share many clinical and epidemiological characteristics, suggesting a potential mutual causation (15).

Epidemiology

The epidemiology of IBD varies greatly worldwide and there are also differences between UC and CD. The incidence rate of UC varies between 0.5-24.5/10^5 inhabitants, while the incidence of CD is 0.1-16/10^5 inhabitants worldwide (16). The highest incidence rates are reported in Northern and Western Europe and North America while the incidence is low in Africa, Asia and South America (16). There was a noticable increase in UC incidence in West European and North American countries between the 1950s and 1990s after which the incidence reached a plateau. A similar increase and stabilisation of the incidence of CD was observed in the same areas 15-20 years later (16). The exceptions are Nordic countries where an increase in incidence is still reported, e.g. in Denmark for both UC (4.1/10^5-8.6/10^5) and CD (9.2/10^5-13.4/10^5) and in Sweden for CD (4.9/10^5-8.3/10^5) (17, 18). In other reports the predominance of UC appears to be diminishing while CD is becoming more prevalent. In a study from Northern France the incidence values during 1988-1999 increased for CD from 5.2/10^5-6.4/10^5 while they decreased for UC 4.2/10^5-3.5/10^5 (19). In Eastern Europe South America and Asia a similar trend with increase in incidence for IBD, as was observed in westernized countries earlier, began in the 1990s. The values for Eastern European countries are approaching the incidence values in the rest of Europe while the values in Asia and South America are still low despite the increase. However there are some exceptions where unexpectedly high prevalence numbers have been reported; e.g. in Northern India the prevalence for UC is 42.8/10^5 and in New Zealand the prevalence is 355.2/10^5 for CD and 145.0/10^5 for UC (16). The observed trends in increase of incidence could in part be explained by improvement of diagnostic procedures which were not available earlier in Eastern Europe, Asia and South America. A greater awareness of the disease could also result in identification of mild cases, previously unnoticed. The increase in severe cases can however not be explained by these factors; it is hence suggested that environmental factors are involved (16). Most studies of CD worldwide have observed a female to male ratio of 1.2:1 and the age of the diagnosed patients are most often between 15 to 30 years. UC affects patients predominantly aged from 20 to 40 years old and women seem to be affected more than men (15).

Pathology

Of patients with UC 20% have total colitis, 30-40% have a disease extending beyond the sigmoid but not involving the whole colon and 40-50% have disease limited to the rectum and recto-sigmoideum. A mild form of UC shows a hyperaemic, oedematous and granular mucosa while, as the disease becomes more severe, the mucosa becomes intensely haemorrhagic and punctuate ulcers become visible. These may extend and deepen into the lam-

ina propria. The ulceration may also extend to the muscle, which can undergo ischemic necrosis.

CD in patients is seen predominantly in the distal ileum and the proximal colon. Nearly half of the patients have a disease affecting both ileum and colon, about one third have disease limited to ileum and some times even including the jejunum while 20-25% have disease confined to the colon. Gross involvement of oesophagus, stomach and jejunum are seldom seen. The earliest lesions in CD are aphthous ulcers which are minute superficial ulcers surrounded by a halo of erythema. Granulomas are characteristic for CD but are neither unique nor universal findings for the disease. When the disease becomes chronic the aphthous ulcers may join together to form larger ulcers that are more stellate. These ulcers can have a longitudinal or transversal form surrounding relatively normal mucosa. Fibrosis can also occur in these patients and can cause hypertrophy of muscularis mucosa.

Clinical presentation

There may be variations in the presentation of CD due to where the disease is located in the gastrointestinal tract, the intensity of the inflammation and the presence of intestinal and extraintestinal complications. The most common complaint from CD patients is diarrhoea. Compared with UC, abdominal pain is more frequent in CD. Faecal occult blood may be found in half of the patients, but it is not as common as in UC and acute haemorrhage is rare. Other symptoms that may be prominent are weight loss, fever and growth retardation in children and these can also be the only detectable symptoms of the disease. The presentation of UC can also vary but the severity of the symptoms is often correlated to the severity of the disease. The common symptoms are diarrhoea, rectal bleeding, passage of mucus and abdominal pain.

Extraintestinal manifestations

An important part of the clinical presentation in both UC and CD patients are the numerous extraintestinal manifestations that can appear. The frequency of such manifestations that have been reported varies between 6-47% (20). The most commonly involved organs are joints, eyes, skin, biliary tract and lungs. These symptoms usually follow the clinical course of IBD but they can precede and even over-shadow the bowel symptoms (20). Primary sclerosing cholangitis (PSC), one of these extraintestinal manifestations, will be discussed further. One suggested explanation for the occurrence of extraintestinal manifestations is the presence of a common autoantigen in the intestine and the affected organs to which an autoimmune attack could be directed. So far one such autoantigen has been suggested and was detected with a murine monoclonal antibody developed against a colon epithelial protein which binds to a cross reactive peptide in eyes (non-pigmented

ciliary epithelium), skin (keratinocytes), joint (chondrocytes), biliary epithelium and the intestine (21, 22).

Aetiology and pathogenesis

When examining the pathologic findings of CD and UC it is clear that IBD is a state of sustained immune response. One key question that arises is whether this response is a normal response towards an unknown pathogen or whether it is an abnormal response to harmless stimulus.

Genetics

When observing IBD populations it is found that first degree relatives of IBD patients have a higher risk of obtaining IBD than the general population with relative risks of ≤35 for CD and ≤15 for UC. (23). The concordance for monozygotic twins and dizygotic twins respectively are 22-64% and 3-4% for CD, and 16-18% and 2-4.5% for UC (24-26). These findings suggest that susceptibility is inherited and that there is a genetic contribution to the development, which is more important for CD than UC. Usually either only CD or only UC affects one family, but there are families with mixed diseases, which implies genetic similarities in the diseases (15). None of the diseases can be explained by a simple mendelian inheritance; therefore it is suggested that multiple gene products contribute to a person's risk of developing IBD.

Screening of DNA from families with multiple affected members identified an area of linkage on chromosome 16, the IBD 1 locus, in CD but not in UC (27). The responsible gene for the linkage was found to be encoding the protein NOD2 (28, 29). Individuals that are homozygous for the disease associated *NOD2* allele can have a 15-40 fold increase of susceptibility to CD (30). NOD2 is expressed in cells of innate immunity as well as epithelial cells and Paneth cells. It is thought to serve as a pattern-recognition receptor for bacterial lipopolysaccharide, i.e. muramyl dipeptide (MDP). This interaction stimulates the secretion of α-defensins and also regulates nuclear factor-κB (NFκB) activation (31). The main CD associated polymorphisms have recently been shown to cause "loss of function" due to an MDP sensing defect (32). These results, observed when studying peripheral blood mononuclear cells may, however, not reflect the function in the intestine.

The genome-wide studies have led to the discovery of several putative susceptibility genes. A variant of the interleukin-23 receptor (IL23R) has been associated with protection for CD (33, 34). IL23 is important for differentiation of Th cells into Th17 cells, which have been shown to mediate chronic autoimmune inflammatory conditions in animal models (1, 35). Thus IL23 has a central role in development of intestinal disease. A single nucleotide polymorphism (SNP) in autophagy related 16-like 1 (ATG16L1) has been associated to CD (36, 37). Autophagy re-processes cell cytoplasmic

ingredients and are also important for inhibiting *Myobacterium tuberculosis* survival in infected macrophages (38).

There is also a genetic background in UC but it is not as evident as in CD. The association between the human leukocyte antigen (HLA) region, which is involved in regulating the immune response, and UC is stronger than the association with CD (39). The association is strongest in patients with extensive UC and includes a positive association with DR2 (in particular the DRB1*1502 subtype), DRB1*0103 and DRB1*12 and a negative association with DR4 and Drw6 (39). The susceptibility genes are probably not located in the HLA region. Another gene associated with UC is the multi drug resistance 1 (MDR1) gene (40). This gene encodes P-glycoprotein 170, an efflux pump of ampiphatic toxins, and is highly expressed on the apical surface on intestinal epithelial cells of the colon and distal small bowel (23). MDR1 knock out mice develop severe intestinal inflammation (41). Several other candidate genes in IBD are involved in the maintenance of the epithelial barrier such as organic cat ion transporters (OCTNs), DLG5 and MyosinIXb, but associations to these genes are inconsistent (23.). TLRs have also been investigated and of these TLR4 is the gene that had the strongest association to development of IBD (31). Although there are many results that support a strong influence of genetic factors in the predisposition of IBD, these cannot solely explain the development of the disease.

Environment

The rise in incidence in IBD might be explained by the effects the environment has on the disease, and several links have been made between environmental factors such as smoking, increased intestinal permeability, diets and drugs (15) and the appearance and progression of these diseases.

Appendectomy is another factor that has been shown to affect development and course of UC. An appendectomy at young age has been shown to be associated with a low risk of subsequent UC (42-44). In another study it was shown that the inverse relationship seems to be limited to patients that undergo surgery before the age of 20 (45). Additionally this study shows a low risk for UC associated with appendectomy performed for an inflammatory condition, but not for an appendectomy performed for a non-inflammatory condition. Further it is suggested that the inflammation preceding the appendectomy is inversely associated with the development of UC rather than the appendectomy itself. A weak positive relation between CD and appendectomy has been reported (46-49).

Microbial factors

An infectious aetiology of IBD caused by a single microorganism has often been suggested but no specific infective candidate has been proven to be the cause of IBD, yet (15). One candidate that has long been suggested to cause CD is *M. paratuberculosis.* A recent publication has shown that CD patients do not benefit from treatment for infection by this bacterium indicating that

it is not involved in the pathogenesis. (50). These results have been questioned due to inadequate testing of presence of the bacteria and the study design (51). Another microorganism investigated is an adherent invasive form of *E. coli* that colonizes the ileal mucosa of CD patients. Whether this *E. coli* indirectly causes CD or whether it is a secondary invader in inflamed mucosa is still uncertain (52). While the harmful pathogens do not seem to cause IBD, loss of immunological tolerance to the harmless autologous bacterial flora is considered to be involved in the development of IBD in humans (53). A study suggesting lack of tolerance to autologous antigens in the IBD patients, showed that lamina propria mononuclear cells (LPMCs) derived from inflamed tissue from IBD patients proliferated in response to both autologous and heterologous sonicated microflora from the intestine, whereas LPMCs from normal individuals only responded to bacterial sonicates from heterologous intestine (54). It is shown in most mouse models that they can develop UC when exposed to non-pathogenic normal colonic bacterial flora but not when they are in a sterile germ-free environment. In the SAMP1/YItFc mice that spontaneously develop IBD the commensal flora seem to exacerbate rather than directly cause the disease, which could also be the case in humans (53). The studies in humans that seek to define whether the intestinal flora in IBD is normal or abnormal are inconclusive so far (53).

Inflammatory factors

The immune system is clearly involved in the pathogenesis of IBD although the immune response is different in UC and CD. It has been shown that in CD and UC different groups of CD4+ T cells are activated, the T_H1 cells and the T_H2 cells respectively. CD is associated with the cytokines produced by the T_H1 cells. The cytokine profile of UC is more unclear at an early stage of the disease but in an established disease the response more closely resembles the T_H2 response (55-57). It has been shown that the main abnormality leading to inflammation is an exaggerated T cell response that causes mucosal hyper-responsiveness to commensal bacteria. The peripheral blood cells and colonic lamina propria CD4+ T cells from CD and UC patients have been shown to cross react with indigenous flora, which suggests that abnormal T-cell specific responses to host flora are important in the pathogenesis of IBD (58).

Although the adaptive immune system mediates the tissue damage, several recent findings indicate that the innate immune system is a prerequisite for the excessive activation of the adaptive system. The innate immune response to intradermal *E.coli* injections and trauma to the skin and intestine was reduced in CD patients but not in UC patients. This suggested a defect in the acute response which was not dependent on *NOD2* mutations. (59)

Mucosal dendritic cells (DCs) which are the main antigen presenting cells in the gut, display an activated phenotype in IBD tissues indicating a role for them in the chronic inflammatory reaction (60). Immature DCs in mice have

been shown to produce IL-23 in response to TLR ligands which contributes to intestinal inflammation in murine models (61, 35). In intestinal epithelial cells TLR3 is down-regulated in active CD but not UC while TLR4 is up-regulated in both UC and CD (62). Moreover, α-defensins are reduced in ileal tissue in CD patients regardless of the degree of inflammation (63). These results and the associations with genes that are part of the innate immune system indicate that this system is important for the development of IBD, especially CD.

As mentioned earlier Th17 is a quite recently identified cell type that has been suggested to play a role in the development of IBD. IL-23 that is needed for the maintenance of IL-17 production by these cells is produced both by activated DCs and macrophages (64). IL-10 deficient mice develop colitis spontanously, however this was prevented by a cross with IL-23p19-deficient mice (65). This result as well as observations in transgenic IL-23 depletion of mice suggests that IL-23 is the main mediator of intestinal inflammation in murine models (65, 35). The associations of IL-23R gene polymorphisms to IBD as well as the detection of an increased number of IL 17^+ cells in inflamed mucosa of patients with active CD and UC suggests that IL-23 signaling and Th17 cells are important also in humans (33, 34, 66).

The defect function of regulatory cells, i.e. failure to suppress or control the immune system, may explain the development of the disease. A mouse model where mice that lack T and B cells (RAG-/-) was induced with colitis after transfer of CD4+ $CD45RB^{high}$ T cells had many of the characteristics of IBD and was Th1 mediated. This disease was successfully prevented by co-transferring of CD4+ $CD45RB^{low}$ T cells that include CD4+CD25+Foxp3+ T cells (T regs) and the T regs could also cure the disease when injected several weeks after induction (67). This effect of T regs has been shown in many murine studies. In IBD patients the effect of T regs is not clear. Two studies have shown that T regs are decreased in peripheral blood from patients with active disease in IBD and in UC respectively (68, 69) This suggests that there is a depletion of T regs which could lead to failure of immune regulation. In two studies of IBD patients and three studies of UC patients it has been observed that the number of T regs is increased in inflamed mucosa during active disease and, where investigated, these cells were functional in vitro (68, 70-73). This suggests that their suppressor effect is affected in vivo or that they are insufficient to control the inflammatory cells.

Autoimmunity in IBD

When the humoral immunity of IBD has been studied much of the interest has been focused on whether disease causing autoantibodies are produced. As early as the late 1950s, an antibody that cross-reacted with colonic cells was found in the serum of UC patients (74). This anti-colon antibody did not have a cytotoxic effect on the colonic cells but this was the first report that suggested that autoimmunity may have a role in the pathogenesis of IBD.

Several autoantigens have been found in the epithelial cells of the gut (75) and both IBD patients (76) and their relatives (77) show sensitization to these antigens.

The antigen that has been best described so far is a 40-kilodalton (kD) colonic epithelial protein that is exclusively recognized by immunoglobulin G (IgG)-antibodies from colon affected by UC (22). Monoclonal antibodies against this antigen have identified a shared epitope in human colon as well as skin, biliary epithelium, eye and joints, which are all locations of extraintestinal manifestations of the disease (21, 22). The autoantigen has been identified as one of the human tropomyosin family of cytoskeletal proteins (78), more exactly isoform 5 (hTM5) (79). Antibodies produced in the mucosa directed to hTM5 were detected in 91% of UC patients (80). Antibodies from UC patients to hTM5 have been shown to destroy colonic epithelial cells in vitro by complement mediated lysis (81). It has also been shown that T cells from peripheral blood and lamina propria in UC secrete IFN-γ when cultured with recombinant hTM5 exceeding the response in CD and healthy controls (82). The relevance of this antigen in the pathogenesis of UC is, however, not yet established.

Autoantibodies against goblet cells have been described in up to 40% of patients with IBD (83-86) and also in 20% of first degree relatives to IBD patients (84). This incidence in autoantibodies is similar to that seen to pancreatic β-cells in patients with insulin-dependent diabetes mellitus and their relatives, which is well established (87). The significance of autoantibodies against goblet cells is still unknown. The goblet cell depletion seen in affected mucosa of UC patients (88), the mucin abnormalities observed in IBD patients causing a defect mucus layer (89) and the high proportion of IBD relatives that have goblet cell antibodies suggests that these antibodies may have a role to play in the pathogenesis of IBD.

Antibodies against the nuclear periphery of neutrophils, that often have intranuclear foci, are named peripheral anti-neutrophil nuclear antigen (pANNA). These antibodies are present in serum of 40-80% of UC patients, 5-25% of CD patients and 1-3% of healthy controls (90). Thus pANNA may serve as a marker for susceptibility. Many proteins have been suggested as the antigen for pANNA but the main target has not yet been identified (91). Another group of autoantibodies are anti pancreatic antibodies directed to exocrine pancreatic tissue. These are detected in 30% of CD patients, 2-6% UC patients and 0-2% of healthy subjects. The relevance of these antibodies in the pathogenesis of CD is unclear (90).

Recently novel potential autoantigens have been described. Ubiquitination factor E4A (UBE4A) has been identified as a candidate autoantigen in CD. Antibodies to this protein were detected in 46% of CD patients compared to 7% and 3% in UC and healthy controls respectively (92). The study also showed that UBE4A was up-regualted in enteroendocrine cells in inflamed ileal mucosa with CD (92). Autoantibodies against CD13 have been identified in IBD patients that had been infected with human cytomegalovi-

rus while no autoantibodies were found in healthy controls (93). Moreover, anti-Enolase-α antibodies have been observed in 50% of IBD patients compared to 8.5% of healthy controls. The autoimmunity to this heat shock protein is suggested to be the result of molecular mimicry with pathogenic heat shock proteins, or release of Enolase-α after necrosis or apoptosis of epithelial cells in which the expression of this protein is up-regulated (94). Whether these antigens play a role in the immunopathogenesis of IBD remains to be determined.

The occurrence of extraintestinal manifestations in IBD can best be explained by an autoimmune pathogenesis where an antigen present at all sites of manifestation cause the inflammation. A hypothesis has been proposed where gut specific lymphocytes are recruited to extraintestinal locations due to expression of gut-specific vascular adhesion molecules in these tissues (95). One such molecule is vascular adhesion protein 1, which was shown to be up-regulated in inflamed skin and functional in lymphocyte adhesion (96). Effector lymphocytes from the gut in IBD showed binding to vessels in chronically inflamed synovial tissue which was dependent on multiple adhesion receptors including vascular adhesion protein 1 and intracellular adhesion molecule 1 (97). The antigens that lead to induction of inflammation are not known, but they seem to be closely linked to gut inflammation since these extraintestinal diseases usually disappear when the bowel inflammation is controlled (95).

Another support for an autoimmune mechanism is reports of UC occurring in reconstructive surgery neovaginas, where no intestinal bacteria or alimentary antigens are present. These findings argue against the hypothesis that direct exposure to alimentary antigens or intestinal flora is the triggering factor of mucosal inflammation (98, 99). Thus autoimmunity is of great interest as a possible mechanism of IBD development.

Goblet cells, mucins and trefoil factors

Throughout the mucosa of the small and large intestine reside goblet cells. These are highly polarised exocrine cells that are recognized for their apical accumulation of secretory granules. These cells produce and secrete high-molecular-weight glycoproteins called mucins. These proteins are huge with a weight of 1-20 x 10^6 Daltons (100), and consist of a protein core with some heavy glycosylated areas and some sparsely glycosylated areas (101). The glycosylation by O-linked oligosaccharides accounts for 60-80% of the weight and is responsible for many of the mucin-properties (101). When secreted the mucins hydrate and form a gel that constitutes the protective mucus that overlays the epithelial surface. This forms a physical and chemical barrier that protects the epithelium from luminal agents such as enteric bacteria, bacterial and environmental toxins, and some dietary components that pose a threat to the mucosa. The mucins also serve as decoy for bacterial

lectin-like receptors. To synthesise mucins for maintenance of the mucus barrier is the responsibility of the goblet cell.

Goblet cells arise by mitosis from multipotent stem cells at the base of the crypt (102) and migrate to the villous tip and are then sloughed into the lumen. Progression of birth to death takes 2-3 days; thus the goblet cell population undergoes constant replacement (103). Although there are goblet cells throughout the intestinal tract, the number varies. There is heterogeneity in mucin production among the goblet cells which divide these cells into different subpopulations. These populations produce and secrete different combinations of mucins and vary by location along the gut and by level of maturation along the crypt-villus axis. All human colonic goblet cells contain more than one mucin species (103). In total 21 different human mucin genes have been identified (104). The major secretory mucins that form the mucus layer are MUC2, MUC5AC, MUC5B and MUC6 (105). The membrane bound mucins are MUC1, MUC3A, MUC3B, MUC4, MUC 11, MUC12, MUC13, MUC17 and MUC20. Some mucins share characteristics of both groups namely MUC7, MUC8, MUC9 and MUC15 (106). The predominant mucins expressed in the colorectum are MUC1, MUC2, MUC3, MUC4, MUC12, MUC13, MUC17 and MUC20 (101, 104). Some structural diversity also exists within stored mucin granules in the cells; hence immunologic distinctions are present not only between adjacent goblet cells but also among granules of an individual goblet cell (103). In IBD patients, changes in glycosylation and sulphation of mucins have been observed which can impair their protective functions (101). Moreover a decrease in expression of MUC3, 4 and 5B in CD patients compared to controls has been observed (107), and similarly decreased expression of several mucins especially MUC2 and 12 was observed in IBD patients compared to controls (104). This suggests that there is a defect mucus layer in IBD patients which fails to protect the epithelial cells from infectious pathogens.

Trefoil factors (TFFs) 1, 2 and 3 are small proteins (7-12 kDa) with motogenic properties that are secretory products of mucous epithelia (108). They are all up-regulated at sites of mucosal injury and stimulate the repair process. Goblet cells in the large intestine produce TFF1 and 2 which are secreted from the cell and stabilize the mucus layer (109). TFF3 is the only TFF that has been shown to be essential for the restitution of the intestinal epithelium (108). Increased levels of TFFs in serum from IBD patients have been observed indicating that TFFs are up-regulated in IBD and can play a role in mucosal protection and repair (110).

Primary sclerosing cholangitis

Primary sclerosing cholangitis (PSC) is a chronic progressive disorder characterized by chronic inflammation and stricture formation of the intra- and extra-hepatic bile ducts. This disease is an extraintestinal manifestation in IBD and also a disease in its own right.

Epidemiology

The incidence for PSC reported in Northern European countries Canada and the American state of Minnesota ranges between 0.9 and 1.3 per 100,000 and year and the prevalence is 8.5-13.6 per 100,000 (111-114). Most reports of PSC epidemiology are studies performed in North America or North Europe, but studies have been carried out in Spain, Japan and Singapore however the disease is much less frequent in these countries (115-117). An increase in prevalence was detected in Spain from 0.78 cases per million in 1984 to 2.24 cases per million in 1988 (115). It is unclear whether this is a true increase or whether it is due to improved diagnostics. The male predominance in PSC is 2:1 (118).

As described earlier PSC is an extraintestinal manifestation in IBD and is present in 3-4% of patients with IBD, conversely IBD can be found in 62-73% of PSC patients. UC is most commonly associated with PSC but CD has also been associated with 1-14% of PSC patients (119).

Pathology

Primary sclerosing cholangitis (PSC) is a chronic cholestatic disease of the biliary tree. It is characterized by stricturing of the intra- and extrahepatic bile ducts with dilation of the areas in between and concentric obliterative fibrosis of intrahepatic bile ducts eventually leading to cirrhosis (120).

Clinical presentation

The clinical course of PSC is characterized by recurrent episodes of cholangitis, during which the disease slowly progresses. Patients may remain asymptomatic for years or may develop symptoms of fatigue, abdominal discomfort, pruritus, fever, jaundice and weight loss. Ultimately the patients develop liver failure or cholangiocarcinoma. At end stage of PSC liver transplantation is the only possible cure, and most reports demonstrate a patient and graft survival above 80% at 10 years and beyond (121).

Cancer in PSC

Cancer is a complication of PSC which increases the mortality in these patients (120, 122-126). The cancers that PSC patients have an increased risk for are hepatobiliary carcinoma (cholangiocarcinoma, hepato-cellular carci-

noma, and gall-bladder carcinoma) (RR=161), colorectal carcinoma (RR=10) and pancreatic carcinoma (RR=14) (122). These cancers are difficult to diagnose and are therefore often detected at a late stage, either at transplantation or autopsy. Despite several attempts, risk factors for these cancers have not been defined (127-130).

Aetiology and pathogenesis

Similarly to IBD the cause of PSC is still unknown. It is currently considered to be an immune-mediated disease of multifactorial and polygenic aetiology.

Genetics

Several MHC genes are associated with PSC. Some MHC haplotypes are associated with increased risk for PSC, these include MICA*008, DRB1*0301, DRB1*1301 and DDRB1*1501 (119). The strongest association is for MICA*008 homozygocity (odds ratio [OR] 5.01). This allele encodes for MHC class I chain-related molecule A (MICA) which are ligands for the NKG2D receptors present on several immune cells including natural killer (NK) and γδT cells. Interestingly increased numbers of these cells have been observed in PSC livers indicating a causal relation to the MICA allele (131). Other MHC haplotypes are found in lower frequencies in PSC patients compared to control, such as DRB1701, DRB1*0401 and MICA*002, and hence are suggested to be protective haplotypes (119).

Other genes have also been associated with PSC but they have often not been replicated. One such example is the 32bp-deletion of the chemokine receptor 5. This deletion results in a reduced receptor expression on T cells and is frequently found in North European countries. A Belgian study showed that there was a significantly lower frequency of this mutation in PSC patients compared to healthy controls, suggesting a protective effect (132). In contrast, an Australian study showed that the frequency of this deletion was higher in PSC patients (133). The Belgian group has however continued with investigation of CCL5, one of the ligands of chemokine receptor 5 and found that the promoter polymorphism -28G was significantly more frequent in PSC patients compared to IBD and also significantly more frequent in CD patients that developed PSC compared to the CD patients who did not (134). Other examples of genes with conflicting results are *E469E* homozygosity of the intracellular adhesion molecule-1, which was associated with protection against PSC and the cystic fibrosis transmembrane conductance regulator (CFTR) in which mutations and variants were shown to be more common in PSC. These results were not replicated in other studies (119).

Knock out of the multidrug resistance (Mdr) 2 gene in mice results in disrupted tight junctions and basement membranes, bile acid leakage and PSC like lesions (135). For the corresponding human gene *MDR3* (ABCB4) and

the bile salt export protein *ABCB11* however, there are no differences in haplotypes in PSC patients compared to healthy controls (136).

Microbial factors

The link between IBD and PSC has resulted in the suggestion that the inflammatory response in the intestine leads to increased permeability and translocation of bacteria to the biliary tree via the portal circulation (137). A study supporting this theory reported positive bacterial cultures in explanted livers from 21 of 36 PSC patients compared to none in livers explanted from 14 Primary biliary cirrhosis (PBC) patients (138). Another study however reported no significant bacteraemia in either portal or systemic blood in 8 UC patients undergoing surgery for severe uncontrolled disease (139). In concordance with this theory bacterial overgrowth of the small intestinal in a rat model leads to biliary and portal inflammation (140), however, in humans this does not seem to be the case. In a study on 22 PSC patients the intestinal permeability was normal in all patients and only one patient had bacterial overgrowth in the small intestine (141).

Specific infectious agents that have been suggested to be involved in the pathogenesis of PSC are helicobacter species, cytomegalovirus and reovirus but results are conflicting and collectively there is no support any of these agents cause PSC (131). Enteric bacteria have been detected in bile obtained during endoscopic retrograde cholangiopancreatography, but this was found primarily in patients who have previously undergone this procedure or who have dominant stenoses indicating that these infections are more relevant for progression rather than the pathogenesis of PSC (119).

Autoimmunity in PSC

PSC, like IBD, is often considered to be an autoimmune disease (137), and these diseases have been suggested to have a common aetiology linked by a shared autoantigen as described above (22). The binding to the bileduct of the mouse monoclonal antibody developed against a colonic epithelial protein was blocked by pre-incubation with 63% of PSC sera but not by control sera (142). The autoantigen has been identified as hTM5 as described above. Autoantibody response to a peptide with a specific sequence of TM has been investigated and all of the 31 PSC patients were positive compared to 69% of UC patients and 5% of healthy controls (143). There are however no studies of possible effector cells directed to hTM5 in biliary epithelial cells. Another biliary epithelial cell autoantigen, against which antibodies was detected in 63% of PSC patients compared to 37% of PBC patients, 16% of autoimmune hepatitis (AIH) patients and 9% of healthy controls, was identified as a 40-kD protein. Furthermore, only antibodies from PSC patients directed to this antigen induced high levels of IL-6 production in the biliary epithelial cells, as well as expression of adhesion molecule CD44 (144). A later study showed that the binding of these antibodies to biliary epithelial

cells initiates ERK1/2 signalling and up-regulation of TLR which upon ligations induces BECs to produce cytokines and chemokines that could lead to recruitment of inflammatory cells (145). These results suggest a link between innate and adaptive immunity in PSC.

A number of autoantibodies in patients with PSC have been described, such as p-ANNAs that are present in 88% of PSC patients; however the target has not been definitely identified (90). Other antibodies present in PSC patients that are considered nonspecific are anti nuclear antibodies, anticardiolipin antibodies, anti-smooth-muscle antibodies, anti thyroid peroxidase antibodies and rheumatoid factor (131). The involvement of humoral immunity is also indicated by observations of elevated circling immune complexes (146), as well as complement activation with elevated C3d and C4d in PSC compared with obstructive cholestasis (147). None of the antibodies with their respective antigens can fully explain the aetiology of PSC and this implies that there might be subgroups of PSC with different pathogenic mechanisms.

Whatever the antigen is, it needs to be recognized by a T cell receptor for activation of cellular immunity. In PSC patients, it has been reported that the Vβ3 T cell receptor gene is predominantly expressed in the hepatic T cells, suggesting that they recognize a specific antigen in the liver which drives T cell expansion (148). Moreover, there is a T cell predominant portal infiltrate in PSC with CD4 cells localized to the portal tracts and CD8 cells at sites of necrosis (149).

The hypothesis that gut-specific lymphocytes are recruited to extraintestinal locations has been investigated also in PSC. In contrast to many other manifestations, PSC can arise independently of inflammation in the gut and is not affected by surgical removal of the colon. These facts raised the hypothesis that PSC is mediated by long-lived memory T cells, originally activated in the gut, which are able to mediate extraintestinal inflammation in the absence of active IBD (95). The support for this hypothesis comes from a number of observations. Mucosal addressin cell adhesion molecule-1 (MadCAM-1), which is normally restricted to the gut, is expressed in liver endothelium in inflammatory liver diseases that are associated with IBD (150). MadCAM-1 also supports *in vitro* $\alpha_4\beta_7$-integrin-mediated lymphocyte adhesion to the liver endothelium (150). Reversely, expression of vascular adhesion protein-1, which is high on normal liver endothelium but low in normal mucosa, is increased in IBD (151). Livers of patients with PSC showed strong expression of CCL25, a chemokine normally expressed only in gut and thymus, and this was associated with recruitment to the liver of mucosal lymphocytes that express CCR9 and $\alpha_4\beta_7$-integrin (152). This suggests that T cells activated in the gut during episodes of active IBD differentiate into effector cells that can bind to both mucosal and hepatic endothelium. These cells can enter the liver under non inflamed condition via interaction with vascular adhesion protein-1 and some of them will revert to long-lived

memory T cells that can re-circulate to the liver and trigger hepatic inflammation under the right conditions, even in the absence of gut inflammation (152).

Some facts cast doubt on the role of autoimmunity in PSC namely the male preponderance and its non-response to immunosuppressive treatment (131).

Candidate autoantigens

Complement component 3

Complement component 3 (C3) is a protein of 185kD which is formed by an α- and a β-subunit and interacts with at least 25 different soluble and membrane bound proteins (153). This protein is a component necessary for both the classical and the alternative pathway of the complement system and thus has a possible role in the pathogenesis of IBD. Elevated levels of complement C3 in serum has been detected in both CD and PSC patients (154, 155). Further, it has been shown that complement C3 is locally expressed in cells in the intestine of patients with CD, suggesting that production of complement is locally regulated, and that the complement activation contributes to the inflammatory effect (156, 157). In the study by Laufer et al complement C3 mRNA was not detected in intestinal epithelial cells in histological normal tissue, but in diseased specimens there was a distribution of complement C3 mRNA in the epithelial cells of the crypts but not of the villi (156). A study by Ahrenstedt et al. however showed that C3 levels was significantly higher in CD patients compared to controls when jejunal-fluid concentrations were measured in a closed segment of the non-affected jejunum (157). There are reports of antibodies to complement factors such as C1q, the C1 inhibitor as well as the C3/C5 convertase (C3bBb) (158). Antibodies to the C3/C5 convertase are called C3 nephritic factor and can be found in sera from patients with membranoproliferative glomerulonephritis or partial lipodystrophy. In the majority of patients the antibody binds to the Bb part of the convertase but reactivity to the C3b part has also been described in 1 of 10 patients with C3 nephritic factor (159).

Glutathione S-transferase theta 1

Glutathione S-transferase theta 1 (GSTT1) is a member of the Glutathione S-transferase (GST) family and is a homodimeric enzyme with subunits of 25 300 Da each (160). In humans there is a genetic polymorphism in the *GSTT1* gene that results in the lack of functional GSTT1 enzyme (161). The genotype with the homozygous deletion of the gene is called "*GSTT1*-null"

while the genotype where at least one copy of the gene is present is called *GSTT1*-positive. In Caucasians the frequency of *GSTT1*-null genotype is about 20% (162-166) while it varies between 11 and 64% in other ethnic groups (167). In the IBD population one study of the null genotype has reported 34.6% of the patients with total colitis was found to have the *GSTT1*-null genotype as compared to 17.5% of those with distal colitis only, 15.6% for CD patients and 17.7% for controls (168). A more recent study from India reported the null-genotype in 90.5% of UC patients and 90% of CD patients compared to 15.9% in healthy controls (169).

In humans GSTT1 is expressed in erythrocytes, lung, kidney, brain, skeletal muscles, heart, spleen and in the gastrointestinal tract (170, 171). In the gastrointestinal tract it is expressed in various cells in the tissues from the oesophagus to the colon including the pancreas, liver and bile ducts. Of special interest regarding IBD is that the protein is expressed in the goblet cells of the colon (172). Like all GSTs, GSTT1 catalyzes the conjugation of glutathione with different species of electrophilic compounds. GSTs are an important part of the cellular detoxification systems and are also thought to protect cells against reactive oxygen metabolites (173).

Autoantibodies directed against GSTT1 have been reported in patients with *de novo* immune hepatitis (174, 175). These autoantibodies are suggested to be produced as a result of an antigraft reaction when a liver or a kidney from a *GSTT1*-positive donor is transplanted into a patient with the *GSTT1*-null genotype. Of the *GSTT1*-null patients that received a liver graft from *GSTT1*-positive donors 12 out of 15 developed GSTT1 antibodies and 6 of these developed *de novo* immune hepatitis. It has not been proven that the autoantibodies mediate the reaction against the graft that results in typical histological features of AIH (175), but it is a possibility. Similarly, *GSTT1*-null patients that receive a *GSTT1*-positive kidney have been reported to develop GSTT1 antibodies (176). A recent study has reported four such patients with kidney biopsies showing pathologic lesions compatible with chronic antibody mediated rejection along with C4d deposition in three of these patients. All of the patients had GSTT1 antibodies that were present before the rejection, whereas three of these patients did not have donor specific anti-HLA antibodies suggesting that GSTT1 antibodies have a role in the anti-graft immune response (177). Furthermore GSTT1 antibodies have also been found when screening patients with suspected or certain autoimmune disease. Screening of about 90,000 patients led to the detection of 18 patients with GSTT1-antibodies. Seven of these patients have been genotyped and they were of the *GSTT1*-null genotype. All of the patients have had either a blood transfusion or have been pregnant with a *GSTT1*-positive child (178). This possible exposure of GSTT1 to these *GSTT1*-null patients is thought to be the cause of the development of the GSTT1 antibodies.

PDZ domain containing 1

PDZ domain containing 1 (Pdzk1) is one of the four members in the family of adaptor proteins that binds Na^+ H^+ Exchanger 3 (NHE3) and are called NHE3 regulatory factors. Pdzk1 is also known as NHE3 regulatory factor 3 (NHERF-3) (179). The members of this protein family have two or four PDZ domains of which many have high identity with PDZ domains of other family members. PDZ domains are the most commonly used human protein-protein interacting sequences. The NHERF-family are adaptor proteins that bind to various proteins that contain PDZ domain ligands and can thereby facilitate protein-protein interactions (179).

Pdzk1 has a size of 63 kD and is expressed in kidney, pancreas, liver, gastrointestinal tract and adrenal cortex (180). Pdzk1 can form heterooligomers with NHERF-1 in vitro, which suggests that a network of PDZ adaptor proteins could form (181). Pdzk1 is located to the apical membrane of epithelial cells by binding to membrane-associated protein 17KD (182). The scaffold protein function of Pdzk1 allows it to locate other proteins to the membrane by binding to them, for example nitric oxide synthase-2 (183). Pdzk1 also interacts with and regulates the function of OCTN2 (184), which is particularly interesting since mutations in the gene coding for OCTN2, *SLC22A5*, are associated with increased susceptibility to IBD (185, 186). Impaired function of OCTN2 caused by mutation or an immune attack would lead to reduced transport of carnitine causing impaired fatty acid β-oxidation in the intestinal epithelium, which is exacerbated by bacterial metabolites and causes colitis in an experimental model (187).

Apart from NHE3, Pdzk1 also binds other proteins involved in the gastrointestinal ion transport such as CFTR and members of the SLC26 apical anion exchange family (188). Pdzk1 appears to mediate the dimerization of CFTR which increases the probability of channel opening (189). Binding of CFTR and SLC26A3 to PDZ adaptor proteins is necessary for the activation of CFTR by SLC26A3 (190). PDZ adaptor proteins also seem necessary for interactions between SLC26A3 and NHE3 as well as NEH3 and CFTR but which of the PDZ adaptors that are involved is not fully elucidated (188). Dysregulated ion transport in the gastrointestinal tract involving Pdzk1 have been suggested to have implications in secretory diarrhoea in IBD (191) and dysregulation in the biliary tract would probably affect secretion in this tissue.

Current investigation

Paper I

Aim

The presence of antibodies in IBD patients directed to the gut and specifically goblet cells have been described previously but a corresponding autoantigen mediating tissue injury has not yet been identified. We have set out to identify such an autoantigen and as a first step we wanted to investigate the presence of autoantibodies against normal intestinal mucosa.

Results

Normal human tissue from the whole gastrointestinal tract (from gastric corpus to rectum) was stained using IBD sera from 14 patients as a primary antibody. All 14 patients had antibodies against goblet cells at some level of the intestinal tract but none of the 20 controls did. The intensity of the immunoreactivity to the mucous substance of goblet cells with sera from IBD patients positive for goblet cell antibodies varied from weak to strong. The number of immunoreactive cells and the intensity of the immunostaining varied between different sera, and between the different regions of the intestinal tract. A correlation between immunoreactivity against goblet cells and the clinically affected segments could be observed for most patients. Staining specific for gastrin producing endocrine cells in the antrum and proximal duodenum was also observed in 3 of 14 IBD patient sera.

Interestingly all of the patients, both UC and CD, had antibodies against goblet cells in the appendix (Fig. 2), hence this tissue was stained with sera from an additional 36 IBD patients (50 in total) and 30 healthy controls (50 in total) and also with sera from 20 patients with celiac disease and 21 patients with infectious gastroenteritis. In total, sera from 22 of the 25 patients with UC (88%) and 20 of the 25 CD patients (80%) showed immunoreactivity against goblet cells in the appendix, while only 4 out of 50 (8%) healthy controls showed immunoreactivity. In the control inflammatory diseases celiac disease and infectious gastroenteritis, 40% and 52% respectively showed immunoreactivity against goblet cells.

The immunoreactivity seen when staining appendix with sera from patients with celiac disease and infectious gastroenteritis differed in compari-

son with IBD serum regarding the intensity of the staining and the staining pattern of the goblet cells. Of the 8 sera from celiac patients that were positive for goblet cell staining 5 where weaker all over and showed less positive staining in the crypts compared to IBD sera. Of 11 of the positive gastroenteritis sera 10 had an all over weaker staining of goblet cells in the appendix and 8 of these also stained fewer cells in the crypts compared to IBD sera.

Staining of 4 colonic biopsies with 8 IBD sera showed weak immunoreactivity to the remaining (Periodic acid-Schiff's positive) goblet cells with two of the IBD sera in two of the biopsies. These biopsies were from patients with inactive and moderately active UC, respectively. In the remaining UC biopsies with moderate and severe activity no immunoreactivity was detected (Table 1).

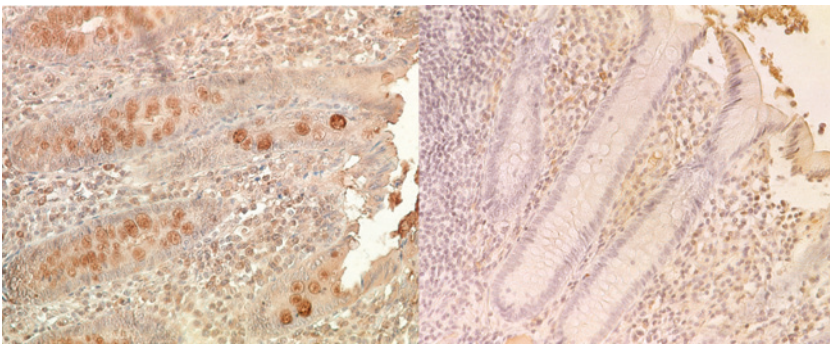

Figure 2. Immunostaining of normal appendix using sera diluted 1 in 100 from a patient with IBD (left) and a healthy blood donor (right). The immunoreactivity to goblet cells is observed in all crypts for the IBD serum, whereas staining of goblet cells is absent using blood donor serum

Discussion

The incidence of antibodies in this study is higher compared to earlier studies, 84% compared to up to 40% that has been reported previously (83-86). This could be due to a difference in the choice of tissue and immunohistochemical technique. In this study we use human tissue from different parts of the gastrointestinal tract whereas other studies have used tissue from other species, for example monkey, usually taken from only one region, most often the ileum (84). The specificity of goblet cell antibodies in this study is high (92%) for distinguishing IBD patients from healthy controls but lower (50-60%) when comparing IBD to the control gastrointestinal inflammatory diseases. The differences in staining pattern between IBD and non-IBD controls, however, suggest that there are different targets for the immunoreactivity in these diseases.

Table 1. Histological inflammatory score, GC characteristics and GC staining using eight IBD sera on four colon biopsies from UC patients and normal control mucosa.

. Colon biopsies	**Histological score**	**GC morphology**	**Mucin depletion**	**Staining by UC sera**
Normal mucosa	6	Normal	None	7/8
Inactive UC	9	Normal	None	2/8
Moderately active UC	15	Normal	Mild	2/8
Moderately active UC	14	Normal	Moderate	0/8
Active UC	18	Normal	Severe	0/8

The high immunoreactivity of IBD sera to the appendix (84%) suggests that the appendix might be of special importance in the development of IBD. The appendix seem to be of central importance for the development of the mucosal immune function with a high proportion of immune cells both of the T- and B-cell lineage (192, 193). This is also consistent with the finding that removal of an inflamed appendix at early age might be protective for UC but not CD (194, 195). Appendectomy may reduce the exposure of goblet cell autoantigens to the immune system, and hence reduce the risk of the development of an immune reaction against goblet cells.

Strikingly only two IBD sera showed weak immunoreactivity to goblet cells in biopsies with no or mild mucin depletion whereas no goblet cell reactivity was seen in biopsies with moderate and severe mucin depletion, when stained with IBD sera. This suggests that the remaining goblet cells have a reduced expression of the antigen in the disease state or that the antigen containing goblet cells have been destroyed through an immune attack.

The results of this study suggest that autoantibodies directed to goblet cells could be important in the pathogenesis of IBD. The identification of the autoantigen(s) to which the immunoreactivity observed in this study is directed, is essential to explain the importance of autoantibodies to goblet cells in IBD. If a putative antigen to the goblet cell antibodies is found, these antibodies could be used as a diagnostic marker. Furthermore the pathogenesis of IBD could be better understood and perhaps lead to new therapeutic possibilities for the IBD patients.

Paper II

Aim

Immunoreactivity to goblet cells has been described in up to 84% of patients with IBD where antibodies bind to mucus in these cells but the corresponding autoantigen has not been identified. The aim of this study is to identify such autoantigen(s).

Results

The immunoreactivity of specific antibodies for candidate autoantigens (MUC1, MUC2, MUC3, TFF1, TFF2, TFF3, adenomatous polyposis coli protein (APC) and three glycosylating enzymes) was investigated in appendiceal tissue as well as goblet cell-like cell line HT29-mtx and compared to the immunoreactivity observed using IBD sera. Staining patterns were similar for IBD sera and antibodies to TFF3 as well as APC, however fluorescent double staining showed that IBD sera did not co-localize with either of these specific antibodies (Fig. 3). In accordance with these results pre-incubation of IBD sera with TFF3 dimer did not affect the staining pattern in appendiceal tissue.

A cDNA library from human appendix mRNA was constructed and screened twice, with serum from one patient with CD and one with UC. The screening resulted in the identification of 48 individual clones of which 3 were identified as complement C3, also 4 single clones of specific interest were identified, namely; calnexin, SON DNA binding protein, nucleoporin and supervillin. *In vitro* translated proteins labelled with ^{35}S-methionine was used for immunoprecipitation with sera from patients and controls. The results for the four single clones showed no difference in immunoreactivity between patients and controls for these proteins. For complement C3, a marked difference in immunoreactivity between patients and controls was detected. Fifteen out of 65 (23%) of the IBD patients and 7 out of 17 (41%) of the PSC patients was above the cut-off level and all healthy blood donors were negative.

The immunoreactivity to granules in the cytoplasm of HT29-mtx cells was present when staining with all of the six IBD sera tested compared to negative or very weak staining with sera from healthy blood donors, indicating the presence of autoantigenic structures in HT29-mtx cells. Immunoprecipitation of metabolically labelled proteins from HT29-mtx cells with sera from patients with IBD and healthy blood donors followed by separation on SDS-PAGE revealed several protein bands specific for IBD patients. The bands to which the immunoreactivity in IBD patients was most frequent were of sizes >200kD, ~93kD and ~60kD.

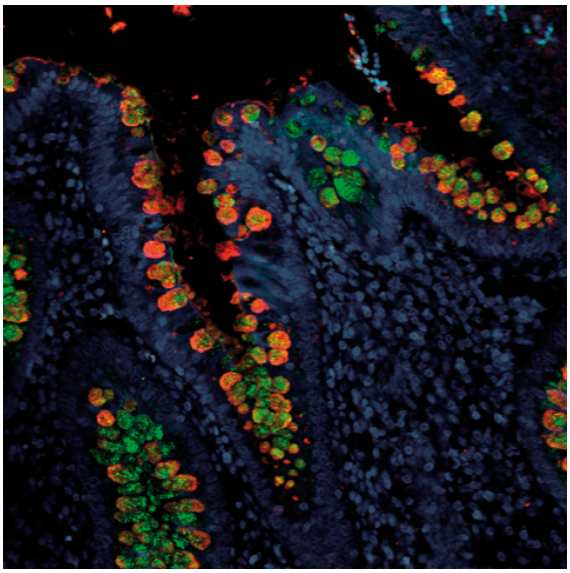

Figure 3. Double immunoflourescent staining of normal appendix using IBD patient sera (red) and monoclonal antibody to TFF3 (green). DAPI (blue) was used for staining of nucleuses. Antibodies against TFF3 and serum antibodies do not co-localize.

Immunoprecipitation of proteins from HT29-mtx cells with sera from two IBD patients and one blood donor followed by 2D gel electrophoresis resulted in identification of 18 spots that were specific for the IBD patients. Nine of these spots were identified using mass spectrometry. Five of the 9 proteins are not present in goblet cells of the colon according to data from the Human Protein Atlas (www.proteinatlas.org). The remaining candidate proteins were enolase 1, hemoglobin beta, gelsolin and. apo-cellular retinoic acid binding protein

Discussion

The candidate approach suggests that none of the investigated proteins are targets for the antibodies directed against goblet cells that are present in sera from IBD patients.

The identification of complement C3 as an antigen in 23% of IBD patients and 41% of PSC patients is interesting. Antibodies to other complement factors have been described earlier (158) but antibodies to complement C3 are, as far as we know, a novel finding. Further studies are needed to validate and explore this finding.

The immunoreactivity of IBD sera to cytoplasm granules in HT29-mtx cells indicates that autoantigens present in goblet cells in the appendix are also present in this cell line. The identification of several proteins specifically precipitated with IBD sera suggests that there might be several autoan-

tigens that are responsible for the immunoreactivity observed in goblet cells. Protein bands with sizes corresponding to the ones detected on SDS-PAGE in this study have been observed earlier as a result of immunoprecipitation of proteins from colon cancer cell lines with IBD sera, which supports the role for these proteins as possible autoantigens (196-198).

The identification of α-enolase, by usage of 2D gel electrophoresis and mass spectrometry analysis, as one of the proteins specifically precipitated with IBD sera is in accordance with a recent study where α-enolase was detected as a target antigen for antibodies present in 50% of IBD patients (94). Anti-α-enolase antibodies have been reported in various diseases most chronic inflammatory and/or immune disorders (199). Thus these antibodies are not disease specific but they could be involved in the pathogenesis of IBD. The identification of α-enolase suggests that autoantigens in IBD can be identified by the method used in this study. Further investigation of hemoglobin β, gelsolin and apo-cellular retinoic acid binding protein, as well as identification by mass spectrometry analysis of the remaining proteins specific for IBD, is needed to pursue the aim of identifying the goblet cell antigen(s).

Paper III

Aim

PSC is an extraintestinal manifestation of IBD and a common aetiology with a shared autoantigen has been suggested. PSC is also a disease without good diagnostic markers. We aimed to identify an autoantigen in PSC, which could serve as a marker and possibly also link IBD and PSC.

Results

A cDNA library from human choledochus was constructed and screened with serum from a patient with PSC and UC. Four individual positive clones were isolated and identified as GSTT1. *In vitro* translated GSTT1 labelled with ^{35}S-methionine was used for immunoprecipitation with sera from patients. The sera tested for reactivity against GSTT1 included 58 patients with PSC and 57 patients with IBD. Serum samples from 118 healthy blood donors and from several autoimmune diseases were used as controls, see figure 4. The upper normal limit of the GSTT1 antibody index was set to 9.4 - the mean value for the blood donors plus five standard deviations. The values above this cut off were considered to indicate presence of autoantibodies to GSTT1.

As seen in figure 4, 3 out of 58 (5.2%) of the patients with PSC and 2 out of 57 (3.5%) of the patients with IBD had antibodies against GSTT1. However, also 3 patients (9.7%) with Hashimoto's thyroiditis, 1 patient (3.3%) with primary biliary cirrhosis and 3 (11%) patients with Graves' disease were above the cut off value. Neither of the other patients had autoantibodies against GSTT1 nor did any of the healthy blood donors (Fig. 4).

To determine the presence or absence of the *GSTT1* allele of 15 PSC patients without GSTT1 antibodies and two IBD patients with GSTT1-antibodies, both microarray and PCR methods were used. Both of these methods showed that the two IBD patients that were positive for GSTT1-antibodies were both of the *GSTT1*-null genotype. Moreover 3 out of the 15 patients without GSTT1 antibodies had the *GSTT1*-null genotype. Two of these patients had had blood transfusions or transplantations and they had all been pregnant. Out of the patients with GSTT1 antibodies, for those of whom we had the information, all had had transplantation, blood-transfusion or had been pregnant.

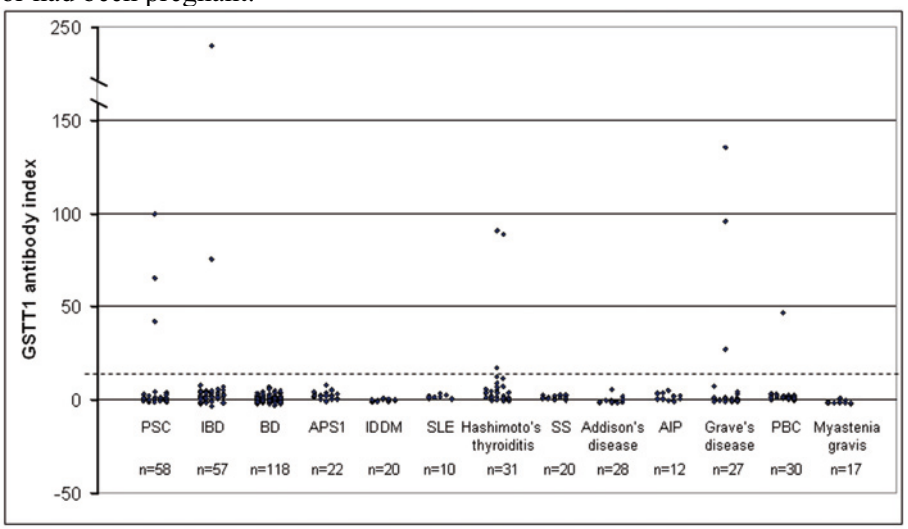

Figure 4. Immunoreactivity to GSTT1 of serum samples from all patients and healthy controls tested in this study. The dashed line indicates the cut off value for positive results, mean value of negative controls plus 7 SD. PSC, Primary sclerosing cholangitis; IBD, Inflammatory bowel disease; BD, blood donors; APS1, Autoimmune polyendocrine syndrome type 1; IDDM, insulin dependent diabetes mellitus; SLE, systemic lupus erythematosus; SS, Sjögren's disease; AIP, autoimmune pancreatitis; PBC, primary biliary cirrhosis

Discussion

We have identified antibodies against GSTT1 in patients with PSC, IBD, Hashimoto's thyroiditis, Graves' disease and primary biliary cirrhosis, thus GSTT1 antibodies are neither specific nor sensitive markers for PSC. From the genotyping results we conclude that the antibodies against GSTT1 pre-

sent in the two IBD patients could not cause the disease since they both have the *GSTT1*-null genotype and do not express GSTT1. A possible implication for these antibodies in the disease mechanism would be cross reactivity to a similar protein. No cross reactivity to the most similar protein GSTT2 was observed.

The most likely cause for development of GSTT1 antibodies is an alloimmune reaction to GSTT1 introduced by transplantation, blood transfusion or pregnancy with a GSTT1-positive child. This has been described by Wichmann et al. (178) but the frequency in their study was 18/~90,000 (0.02%) while our frequency was 12/332 (3.6%). These differences could be explained in part by differences in patient material. Wichmann et al. screened patients with suspected or certain autoimmune diseases while we screened only patients with known autoimmune disease. There are also differences in screening methods and our methods might be more sensitive for detecting autoantibodies. In this study, the prevalence of GSTT1 antibodies was significantly higher in patients with autoimmune disease compared to healthy blood donors. We therefore speculate that the autoimmune phenotype could be a risk factor for development of GSTT1 antibodies.

The three PSC patients that were of the GSTT1-null genotype did not have GSTT1 antibodies. A possible explanation for this is that all donors and babies were of the GSTT1-null genotype, but that chance is small ($p=0.000001$). Another reason could be that GSTT1 might not have been presented in a way that mediated immunity in these patients. Perhaps this kind of presentation is more likely to occur in certain susceptible persons.

The development of GSTT1 antibodies in *GSTT1*-null patients may have implications in transplantations and transfusions where genotyping of donor and recipient could prevent the development of antibodies and perhaps decrease the risk for alloimmune reactions to transplants and erythrocytes.

In conclusion, GSTT1 antibodies seem to be caused by an alloimmune reaction in *GSTT1*-null patients presented with the GSTT1 antigen. The high frequency of GSTT1 antibodies in patients with autoimmune diseases suggests that autoimmune phenotype is a risk factor for development of GSTT1 antibodies or that the *GSTT1*-null genotype is overrepresented in autoimmune diseases. Further investigations are needed to increase our understanding of the possible role of GSTT1 autoantibodies in autoimmune diseases.

Paper IV

Aim

PSC is an extraintestinal manifestation of IBD and a common aetiology with a shared autoantigen has been suggested. PSC is also a disease without good diagnostic markers. We aimed to identify an autoantigen in PSC, which could serve as a marker and possibly also link IBD and PSC.

Results

Sections from normal human choledochus, stained with sera from 34 PSC patients and 28 healthy blood donors revealed a specific staining pattern. This was characterized by a strong immunoreactivity all over the section directed against the apical membrane as well as cytoplasmic granules of biliary epithelial cells (BECs) and was detected with sera from 12 PSC patients (Fig. 5). Staining of the choledochus using the remaining PSC and blood donor sera resulted in immunoreactivity ranging from negative to moderate staining of the apical cell membrane and granules.

To identify the corresponding antigen a cDNA library from human choledochus was constructed and screened with serum from a patient with PSC that showed specific immunoreactivity to BECs. Several positive clones were isolated and identified, of which two coded for Pdzk1. *In vitro* transcription and translation was used to produce ^{35}S-methionine-labelled Pdzk1 which was used for immunoprecipitation with sera from 34 patients with PSC and 55 patients with IBD as well as 30 patients with autoimmune polyendocrine syndrome type 1, 12 patients with autoimmune pancreatitis (AIP), 20 patients with insulin dependent diabetes mellitus, 20 patients with Sjögren's syndrome, 22 patients with Graves' disease, 20 patients with Hashimoto's thyroiditis and 20 patients with Addison's disease. Sera from 94 healthy blood donors were used as controls. The limit for a positive result for autoantibodies to Pdzk1 was set to 58.3 – the mean value for blood donors plus three standard deviations. Two out of 34 (6%) of the patients with PSC and 2 out of 55 (4%) of the patients with IBD as well as 4 patients (18%) with Graves' disease, 1 patient (8%) with AIP and 1 healthy blood donor (1%) had antibodies against Pdzk1.

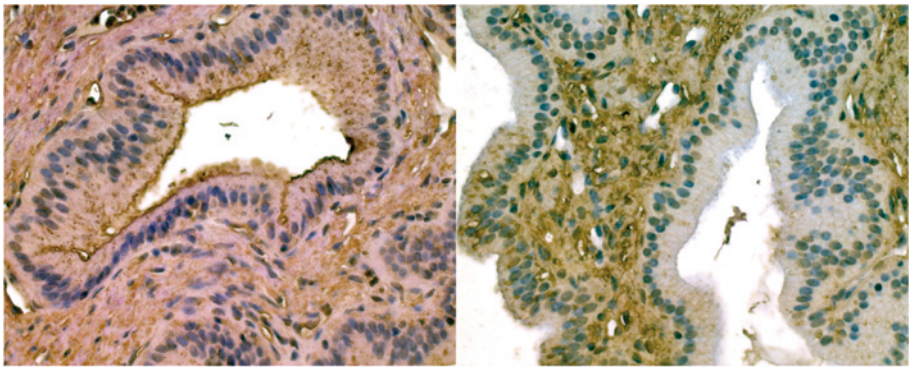

Figure 5. Immunostaining of normal human choledochus using serum in dilution 1 in 100 from a patient with PSC (left) and a healthy blood donor (right). The PSC serum show positive staining with strong immunoreactivity to granules and apical membrane of biliary epithelial cells, whereas the healthy blood donor serum show negative staining with no immunoreactivity to biliary epithelial cells.

Discussion

A novel staining pattern of the BECs in choledochus was observed with strong immunoreactivity to the apical cell membrane and cytoplasm granules. This staining pattern was obtained primarily with PSC sera showing a specificity of 100% and a sensitivity of 35%, indicating that this pattern could be a potential marker for PSC. It remains to be elucidated whether this immunoreactivity is specific for a subgroup of PSC patients.

Pdzk1 was identified by screening of a cDNA library from the choledochus with a serum that produced the novel staining pattern. Pdzk1 is an adaptor protein located to the apical cell membrane and is known to be expressed in BECs of liver tissue and also in epithelial cells of the gastrointestinal tract (180), providing a possible link between these two diseases. Antibodies to Pdzk1 in patients with Graves' disease and AIP and a healthy blood donor can only in part be explained by association with PSC, which implies that these antibodies might not be disease specific.

Only one of the PSC patients with the positive staining pattern had antibodies to Pdzk1; hence the immunoreactivity to the apical membrane could perhaps be caused by Pdzk1 antibodies in some patients. The main target causing this novel staining pattern of BECs, is however yet to be identified.

General discussion and future perspectives

IBD and PSC are both diseases with an unknown aetiology. Currently, the general opinion concerning IBD is that the genetic predisposition, the external environment, the intestinal microbial flora and the immune system are all involved in the generation of IBD (53). Genetic associations in IBD are numerous and many of the identified genes have given new insights to possible pathogenetic mechanisms; e.g. IL-23 that suggests a role for Th17 cells (33, 34), and MDR1 that implies the importance of intestinal membrane transporters and the epithelial barrier function (40). IBD is a disease with a complex genetic background but gene variations alone cannot explain the pathogenesis of IBD.

One widely accepted theory is that inflammation in IBD is the consequence of an abnormal response to autologous gut flora. It is however very difficult to define whether the bacterial flora in IBD patients is normal or abnormal, and it is also not clear whether the flora is the cause of IBD or whether it leads to exacerbation of the disease (53). Specific bacteria have also been suggested as the trigger of disease, but so far none of the candidates have been proved to cause IBD. Moreover, the failure of T regs to suppress the immune system which leads to development of colitis in mice has not been shown in humans (68, 70-73).

None of the theories above can explain the occurrence of extraintestinal diseases in IBD. Such manifestations have been reported in 6-47% of IBD patients and can have a high impact on quality of life, morbidity and even mortality in these patients (20). An antigen present at all sites of inflammation can be the link between IBD and its extraintestinal manifestations, and a possible source for such an antigen are goblet cells which reside at several locations of such manifestations, namely choledochus, nasal mucosa, conjunctiva and bronchus (200). A finding supporting this theory is the development of a conjunctival ulcer with absence of goblet cells in a patient with CD (201), this is in concordance with the goblet cell depletion observed in the colon of patients with UC (88). Antibodies to goblet cells have been described earlier in patients with IBD as well as in their first degree relatives (83-86) and in Paper I we describe goblet cell antibodies in 84% of IBD patients. We also show that the antigen is not present in biopsies with severe or moderate goblet cell depletion which suggests that the cells expressing the antigen could have been destroyed through an immune attack.

A possible mechanism for the pathogenesis of IBD could be initiated by binding of autoantibodies to intestinal goblet cells which leads to an immune attack of these cells, which in turn results in a diminished mucus layer and recruitment of immune cells. This would lead to inflammation as well as a weaker mucosal defence in this area putting it at greater risk of bacterial invasion, which in turn could lead to exacerbation of the inflammation. T cells that are initially activated in the gut could then home to extraintestinal locations by binding to specific adhesion molecules as described by Eksteen et al. and others (96, 97, 152), and upon encountering and binding to the antigen they become activated and can cause inflammation. Another possibility is that bacterial overgrowth and invasion of the intestinal epithelial cells, as the primary injury, leads to exposure of goblet cell antigens, inflammation, production of goblet cell antibodies, and an altered mucous membrane. In this scenario gut specific T cells could also be activated and home to locations of extraintestinal manifestations.

Our attempts to identify an autoantigen that can link IBD and its extraintestinal manifestations have resulted in several candidates. The most promising candidates so far are complement C3 and Pdzk1. On our quest for this autoantigen we also stumbled upon GSTT1, which is not specific for PSC and IBD, however it is an interesting antigen.

Antibodies to complement C3 are as far as we know a novel finding and are present in sera from 23% of the IBD patients and 41% of the PSC patients but none of the healthy blood donors in Paper II. This suggests that antibodies to complement C3 could be a potential marker for IBD and PSC. Elevated serum levels of complement C3 has been detected in patients with CD and PSC (154, 155). Moreover, it has been shown that C3 is locally expressed in cells in the intestine of patients with CD, suggesting local regulation of complement production, and contribution to the inflammatory effect by complement activation (156, 157). Antibodies to other complement factors such as C1q and the C3/C5 convertase (C3bBb) have been described (158) and 1of 10 sera that bind to the convertase has been reported to have some binding to C3b alone (159). Further investigation is needed to determine if antibodies to complement C3 are specific for IBD and PSC and also to which part of complement C3 that the antibodies are binding and if this have some effect on the function of complement C3.

Pdzk1, which was identified using patient sera which also gave the specific staining pattern of BECs, is an adaptor protein which is expressed in epithelial cells and located to the apical membranes in both bile ducts and the gastrointestinal tract (180). Antibodies to Pdzk1 were detected in sera from 6% of the patients with PSC and 4% of the patients with IBD, as well as 18% of patients with Graves' disease, 8% of patients with AIP and 1% of healthy blood donors (Paper IV). Although Graves' disease has been reported in 4% of PSC patients (202) and AIP is associated with sclerosing cholangitis (203), this can not fully explain the high frequency of antibodies

in these patients. This suggests that Pdzk1 antibodies might not be specific for IBD and PSC. Pdzk1 is however an interesting antigen which links the bile ducts and the gastrointestinal tract by expression in epithelial cells (180), and its interaction with several transporter proteins in the intestinal tract implies that it has an important role in the function of the epithelial barrier (184, 188, 189, 191). An interacting protein of special interest is OCTN2 since mutations in the gene coding for this protein have been associated with increased susceptibility to IBD (185, 186). Even though the frequency of antibodies is low in patients with IBD and PSC, this finding could still emphasise the significance of Pdzk1 and its function in the epithelial cells since autoantigens are often proteins with an important function, for example H/K-ATPase in pernicious anaemia (204) and 21-hydroxylase in Addison's disease (12).

GSTT1 is an enzyme that catalyzes the conjugation of glutathione with different species of electrophilic compounds (173). There is a genetic polymorphism in the *GSTT1* gene with a homozygous deletion of the gene resulting in the *GSTT1*-null genotype (161). This genotype is present in about 20% of Caucasians (162-166) and results in the lack of functional GSTT1 (161). Antibodies to GSTT1 have been reported to develop as an alloimmune reaction in patients of the null genotype that have received a liver, kidney or blood transplant from a *GSTT1*-positive donor, as well as in *GSTT1*- null mothers pregnant with a *GSTT1*-positive child (174-176,178). We identified GSTT1 antibodies in 5,2% of PSC patients and in 3.6% of all patients with autoimmune disease investigated which was significantly higher compared to healthy blood donors. All patients had had blood transfusions or been pregnant, and the GSTT1 antibody positive patients that were tested were of the *GSTT1*-null genotype (Paper III). We speculate that the autoimmune phenotype could be a risk factor for the development of GSTT1 antibodies or that the *GSTT1*-null genotype is more common in patients with autoimmune disease. Other studies have reported an important role for genetics in autoantibody susceptibility, for example the high heritability of IgM anti-Ro shown in SLE patients, which is correlated with IgG anti-Ro, IgG anti-La and IgG anti-dsDNA in these patients (205).

De novo autoimmune hepatitis have been reported in patients of the *GSTT1*-null genotype that received a *GSTT1*-positive liver. These patients had antibodies to GSTT1, but it could not be concluded that these antibodies caused the inflammation of the liver (175). Recently it was shown that 3 out of 4 *GSTT1*-null patients that had received *GSTT1*-positive kidneys had kidney biopsies showing pathologic lesions compatible with chronic antibody mediated rejection along with C4d deposition. All of the patients had GSTT1 antibodies that were present before the rejection, whereas three of these patients did not have donor specific anti-HLA antibodies, suggesting that GSTT1 antibodies has a role in the anti-graft immune response (177). At the endstage of PSC, a liver transplant is the only possible cure and considering

the possibility that PSC patients have a higher frequency of the *GSTT1*-null genotype, the genotyping of donor and recipients could be of importance to promote the survival of allografts.

The quest for the antigen that links IBD and PSC will pursue by further investigation of the candidate antigens where the ultimate goal would be to identify and define the B cell and T cell response to an antigen. The importance of identifying autoantigens in autoimmune diseases is illustrated in PBC where the identification of the E2 component of pyruvate dehydrogenase in PBC as a major autoantigen has led to a deeper understanding of the mechanisms underlying the disease (206).

Sammanfattning på svenska

Inflammatoriska tarmsjukdomar, på engelska inflammatory bowel disease (IBD), utgörs främst av ulcerös kolit och Crohns sjukdom. Patienter med IBD har en kronisk eller återkommande inflammation i tarmen vilket beror på ett ihållande ökat immunsvar i denna vävnad. Vårt immunförsvar har till uppgift att skydda oss mot främmande patogen såsom bakterier och virus, men kan också gå till angrepp mot kroppsegna vävnader varvid autoimmuna sjukdomar uppkommer. Vid uppkomst av IBD är det ännu inte fastställt huruvida det ökade immunsvaret är ett normalt svar riktat mot ett patogen eller om det är ett anormalt svar riktat mot ett harmlöst stimulus såsom kroppsegen vävnad eller den normala bakteriefloran.

Den rådande uppfattningen om IBD idag är att en samverkan mellan genetiska anlag, miljöfaktorer, den bakteriella floran i tarmen och immunförsvaret leder till utveckling av sjukdomen. Det finns dock vissa aspekter av IBD som inte kan förklaras av denna modell, nämligen förekomsten av extraintestinala manifestationer som har rapporterats i 6-47% av patienter med IBD. Dessa manifestationer utgörs av inflammation i olika vävnader, de vanligaste är hud, leder, ögon, gallgång och lunga. Manifestationerna kan ha en stor inverkan på livskvalitet såväl som morbiditet och mortalitet hos dessa patienter. Ett exempel på en sådan sjukdom är primär skleroserande kolangit (PSC) som karaktäriseras av inflammation och förträngning av gallgångarna och förekommer hos 3-4% av patienter med IBD. En möjlig patogenetisk mekanism som skulle kunna förklara uppkomsten av IBD och dessa extraintestinala manifestationer är en autoimmun reaktion riktad mot ett kroppseget protein, s.k. autoantigen, som finns både i tarmen och i de vävnader där man har manifestationer. En celltyp där man skulle kunna identifiera ett sådant autoantigen är bägarceller vilka finns i mag-tarm kanalen så väl som i ögon, gallgång och lunga. Bägarceller finns i tarmepitelet längst ut mot hålrummet i tarmen, de producerar mucus som innehåller en rad olika proteiner och släpps ut i tarmen där de utgör en del av den skyddande tarmslemhinnan. En autoimmun reaktion riktad mot bägarceller skulle kunna leda till destruktion av dessa celler och därmed avsaknad av mucus i den angripna delen av tarmen. Detta skulle leda till inflammation såväl som ett minskat skydd mot infektion av bakterier vilket i sin tur skulle kunna leda till ökad inflammation. Vita blodkroppar som binder till detta autoantigen och cirkulerar i kroppen skulle sedan kunna binda till autoantigenet i andra delar av kroppen vilket skulle kunna leda till en autoimmun reaktion i andra organ.

I den här avhandlingen har vi undersökt autoimmun reaktivitet mot vävnad från mag-tarm kanalen och gallgången genom att använda sera från patienter med IBD respektive PSC. Sera innehåller de antikroppar som finns i blodet, vilka producerats av vita blodkroppar som svar på aktivering vid bindning till ett antigen och är alltså en markör för ett immunsvar mot detta antigen. Vi har vidare försökt att identifiera de autoantigen som orsakat denna immunoreaktivitet.

I delarbete I har vi genomfört immunohistokemiska färgningar på frisk vävnad från hela mag-tarm kanalen med sera från 14 patienter med IBD och med 20 friska blodgivare som kontroller. Alla IBD patienter hade antikroppar mot bägarceller i olika utsträckning i tarmen, men inga blodgivare hade antikroppar. Alla IBD patienter färgade bägarceller i blindtarmen och därför färgades just vävnad från blindtarmen med sera från totalt 50 IBD patienter och 50 blodgivare. Immunoreaktivitet hos IBD patienterna var 84% medan endast 4% av de friska blodgivarna hade antikroppar mot bägarceller i blindtarmen. Vid färgning med sera från patienter med IBD av inflammerad tarmvävnad från patienter med ulcerös kolit påvisades ett omvänt förhållande mellan immunoreaktivitet mot bägarceller och inflammatorisk aktivitet. Detta tyder på att det antigen som antikropparna riktas mot inte uttrycks i den inflammerade vävnaden eller att de celler som uttrycker antigenet har destruerats genom en immunreaktion.

I delarbete II har vi använt tre olika metoder för att identifiera möjliga autoantigen, en undersökning av immunoreaktivitet mot kandidat antigen som finns i bägarceller i jämförelse med IBD sera, screening med IBD sera av ett cDNA bibliotek där DNA som motsvarar alla proteiner som finns i blindtarmen finns representerade och immunprecipitering av proteiner från en bägarcellslik cellinje följt av identifiering med hjälp av separation på endimensionella och tvådimensionella geler samt analys med mass spektrometeri. Vi identifierade flera autoantigen av vilka komplementfaktor 3 är mest lovande.

I delarbete IV har vi beskrivit ett nytt färgningsmönster vid immunohistokemisk färgning av frisk gallgång med sera från patienter med PSC där antikropparna binder till epitelceller i gallgången. Denna immunoreaktivitet ses hos 35% (12/34) av patienter med PSC men inte hos någon av 28 friska blodgivare. För att identifiera det autoantigen som antikropparna riktas mot utförs i delarbete III och IV screening med PSC sera av ett cDNA bibliotek där DNA som motsvarar alla proteiner uttryckta i gallgång finns representerade. I arbete IV identifieras PDZ domain containing 1 (Pdzk1) som ett potentiellt antigen och i arbete III identifieras Glutathion S-transferas theta 1 (GSTT1). Pdzk1 uttrycks både i gallgång och tarm och är därför en intressant kandidat som kan koppla samman IBD och PSC. Antikroppar mot GSTT1 har vi detekterat hos patienter med PSC men även hos patienter med andra autoimmuna sjukdomar. Tjugo procent av kaukasier har en defekt GSTT1-gen och uttrycker inte detta protein. Antikroppar mot GSTT1 tros uppkomma när dessa patienter får ett transplanterat organ eller en blodtrans-

fusion från en person som uttrycker proteinet eller om de är gravida med ett barn som uttrycker proteinet.

Sammanfattningsvis har vi detekterat autoimmun reaktivitet mot bägarceller i tarmen och epitel celler i gallgången hos patienter med IBD respektive PSC samt identifierat flera potentiella autoantigen. Dessa fynd stärker hypotesen om en autoimmun patogenes för IBD och PSC.

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