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Food Antigen Sensitivity in Coeliac Disease Assessed by the Mucosal Patch Technique

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Abstract

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A diagnosis of coeliac disease (CD) in adults relies on the presence of a structurally abnormal intestinal mucosa, followed by a clear clinical remission on a gluten-free diet. There is a clear need for a rapid, simple, safe and sensitive method to determine the type and intensity of inflammation in the gut mucosa in clinical practice. The overall aims of our studies were to develop and evaluate a new technique, "the mucosal patch technique", to characterize rectal local inflammatory process after rectal food challenge in patients with CD. **In study 1** we evaluated the potential of the new technique. The technique was well tolerated and easily applied. Pronounced neutrophil and eosinophil involvement in ulcerative colitis (UC) was demonstrated. With the high sensitivity of the technique, low-degree mucosal neutrophil activation could also be quantified in patients with collagen colitis, UC in clinical remission and in patients with irritable bowel syndrome. **In study 2 and 3** the aim was to elucidate the dynamics of the rectal inflammatory response and nitric oxide (NO) production after rectal gluten challenge. We found a pronounced neutrophil activation in coeliac patients after rectal gluten challenge. This activation was apparent 4 hours after challenge and remains for at least 48 hours. A more modest eosinophil activation started 1-2 hours later and remained at least for 48 hours. The biphasic pattern of neutrophil and eosinophil activation after challenge suggests a biphasic inflammatory reaction. The activation of neutrophils and eosinophils precedes a pronounced enhancement of mucosal NO production. Some of our coeliac patients displayed signs of an inflammatory reaction after rectal corn gluten challenge. **In study 4** the aim was to investigate the local inflammatory reaction to gluten and cow's milk protein in CD patients in remission. The findings indicate that not only gluten sensitivity but also cow's milk (CM) protein sensitivity is common in CD. The data support the hypothesis that CM sensitivity may contribute to persistent symptoms in coeliac patients on gluten-free diet.

Keywords: Coeliac disease, Diagnostic instrument, food hypersensitivity, gut pathophysiology, inflammation, nitric oxide, rectal instillation, gluten, corn, milk hypersensitivity, inflammatory bowel disease, irritable bowel syndrome

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Know also that wisdom is sweet to
your soul; if you find it, there is a
future hope for you, and your hope
will not be cut off.

Bible, Proverbs 24:14

To my family;
Ragnheiður Harpa, Örn, Erla and Atli

List of Papers

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

- I Kristjánsson G, Venge P, Wanders A, Lööf L, Hällgren R
Clinical and subclinical intestinal inflammation assessed by the mucosal patch technique.
Studies of mucosal neutrophil and eosinophil activation in inflammatory bowel diseases and irritable bowel syndrome.
Gut 2004;53(12):1806-12.
- II Kristjánsson G, Serra J, Lööf L, Venge P, Hällgren R.
The kinetics of mucosal granulocyte activation after gluten challenge in coeliac disease.
Scand J Gastroenterol 2005;40(6):662-669.
- III Kristjánsson G, Högman M, Venge P, Hällgren R
Gut mucosal granulocyte activation precedes nitric oxide production: studies in coeliac patients challenged with gluten and corn.
Gut. 2005 Jun;54(6):769-74
- IV Kristjánsson G, Venge P, Hällgren R
Cow's milk protein sensitivity is occurring frequently in coeliac disease.
Submitted

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Abbreviations

IEL	Intra epithelial lymphocytes
HLA	Human leukocyte antigens
MPO	Myeloperoxidase
HNL	Human neutrophil lipocalin
ECP	Eosinophil cationic protein
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
UC	Ulcerative colitis
CC	Collagen colitis
NO	Nitrogen Oxide
NOS	nitric oxide synthase
ppb	Parts per billion
tTG	Tissue transglutaminase
EmA	Endomysial antibodies
PPV	positive predictive value
ESPGAN	European Society of Pediatric Gastroenterology and Nutrition
CM	Cow's milk
CD	Coeliac disease

Introduction

Background

It is not known when or where people started to suspect food as a cause of illness. Humans have probably known from the beginning that some food could be either toxic or curative. To support this are the histories of wise people, “witches”, monks, doctors etc using diets and remedies made of special food ingredients. Hippocrates mentions the importance of diet and food in illness in his writings 460-375 B.C. Even in modern days we hear and read about all kind of diets, not only to lose weight but also to gain health and live well. During the 20th century research taught us more and more about the physiology of the gut and the interaction of the gut and food. For example, how we digest and absorb the nutrients in food. But research taught us also about the negative interactions with food, for example intolerance and allergy. In spite of the increased insight into these issues during the last decades we have very limited clinical methods to diagnose food allergy. The foundations of diagnostics still rely mainly on a good patient history and observation of the patients’ reactions to food, elimination and challenge.¹

One of the exceptions to this has been the diagnostics of coeliac disease, a disorder characterized by loss of tolerance to specific dietary peptides in wheat, barley, rye and probably oats. The diagnosis of coeliac disease relies mostly on histological changes in the mucosa of the small bowel before and after gluten free diet.² Taking jejunal biopsies with “Watson’s capsule” or during endoscopy is not always well tolerated. In children and in selected adult cases it is done under general anaesthesia. A search for simpler methods has resulted in various serological markers³ and in the late eighties rectal challenge was introduced.⁴ In these initial studies of rectal challenge with gluten, biopsies were used for analysis.⁴⁻⁶ Taking biopsies and evaluating the histological inflammatory mucosal changes is a well established technique in coeliac disease. But taking biopsies is invasive and the procedure presented by Loft and colleagues for evaluation of rectal mucosal biopsies also needs a controlled morphometric technique for the measurement of intra-epithelial lymphocytes (IEL), a technique that demands computerized image analysis, which is not always available.⁶ During the late eighties studies were done on inflammatory bowel disease and coeliac disease with the perfusion technique.^{7,8} The advantage of intestinal and colonic perfusion techniques over morphological and immunohistochemical studies of biopsy specimens is that

they allow quantitative evaluation of the release of humoral substances and cells in the diseased gut mucosa. For research purpose, on smaller populations, the perfusion technique is unique, but for large clinical studies we have recognized the need for a more simple method that is safe, fast and inexpensive, none the less still reliable for assessing inflammation of the gut mucosa. A method that could be used to evaluate mucosal reaction to food proteins in coeliac disease as well as in other food hypersensitivity reactions, would be important.

Gut immune system

The gastrointestinal tract is the site where the divergent needs of nutrient absorption and host defence meet. It is therefore not surprising that most of the immunological cells of the human body are presented here. The first line in our defense is the gut epithelial barrier, which cannot completely prevent luminal antigens from entering the tissues despite of the tight junctions between mucosal epithelial cells. Antigen and bacteria are allowed to cross the barrier through openings/gaps in follicle associated epithelium that overlies the organized lymphoid tissue⁹ or they are sought/caught by specific immune cells like the antigen presenting dendritic cells and the specific epithelial M-cells.⁹ The gut epithelium itself can also directly sense through receptors, nucleotide-binding oligomerization domain molecules from commensal bacteria and pathogens and alert the host of an infection.¹⁰ Thus, the barrier is a highly dynamic structure that limits but does not exclude pathogens and antigens from entering the tissues. It samples gut antigens (like food and bacterial proteins) for recognition, tolerance or protection.

A very specific equilibrium exists between the immune system, gut wall and the gut bacteria. This equilibrium is tightly regulated to prevent excessive immune responses to foods and gut bacteria. Many immune cells in and beneath the barrier are involved in this control process of recognition, tolerating and defending. The immune cells seen in the normal epithelium and lamina propria of the gut are T-lymphocytes, macrophages, eosinophil granulocytes, mast cells and IgA and IgM antibody producing plasma cells. Many of our important gastrointestinal disorders such as Crohn's disease and coeliac disease are thought to involve disturbance of this fundamental immunological equilibrium of the gut.¹¹ The immune responses seen after contact with an external agent are divided into two main types, the adaptive and the innate system.

The adaptive immune system comprises the B- and T- Lymphocytes who are able to memorize an encounter/meeting with a microbe/antigen and thus adapt to be ready for the next encounter with this same agent in the future, including antibody production. As identification and production of proteins etc is needed in this process it takes several hours to start the system.

Various human cells present a peptide sequence, the human leukocyte antigens (HLA), to enable T-lymphocytes to recognize antigen and discriminate self from non-self. Unlike B-cell receptors on B-lymphocytes that are able to directly bind peptide sequences on antigens, the T-cell receptors of T-lymphocytes can only recognize the peptide sequence after they are bound to HLA molecules. Susceptibility to many autoimmune disease are associated with inheritance of specific HLA genes. Examples of diseases with strong association to certain type of HLA molecules are type 1 diabetes, ankylosing spondylitis and coeliac disease.¹²

The innate system on the other hand is the part of the immune system in mammals that acts directly when there is an invasion of microbes, long before the adaptive system starts to react. It serves as a first line of defence and alerts the adaptive system.¹³ Unlike the adaptive system the innate system does not recognize a specific peptide but rather structures or patterns that identify the “enemy”. The innate immune responses involve neutrophils, monocytes, macrophages, basophils, mast cells, eosinophils, natural killer cells and molecules such as complement proteins, acute phase proteins, and cytokines.¹³

Humoral inflammatory mediators in gut and their use as markers of activated granulocytes

In an inflammatory reaction, innate or adaptive, a cellular infiltration occurs involving a mixture of polymorphonuclear leucocytes, macrophages, lymphocytes and plasma cells. Different mixtures of cells are seen in different inflammatory diseases. These cells are recruited and activated by a signalling system that consists of soluble mediators, released by the cells in the inflammatory process itself. Examples of the humoral mediators well known today are granulocyte granule constituents, cytokines and eicosanoids but new mediators are still being recognized.¹⁴

In our studies we have focused on analysis of some humoral mediators such as myeloperoxidase (MPO), human neutrophil lipocalin (HNL), eosinophil cationic protein (ECP) and histamine known to be released in increased amounts in the gut epithelial mucosa during an inflammatory process in the gut.¹⁵⁻¹⁷ Granulocyte mucosal infiltration is a prominent feature in many inflammatory processes in gastrointestinal diseases like Crohn's disease and ulcerative colitis (UC) but it is also known to be found in gut inflammation in coeliac disease.^{7,17}

Markers of neutrophils

Neutrophils, the most abundant of the circulating white blood cells, can ingest and kill invading microorganisms. Central in this functional role are the neutrophils granule compartments and the granule constituent proteins. Dur-

ing the migration to the place of invasion (diapedesis) and during the phagocytosis (ingestion), granule proteins, reactive oxygen and nitrogen species are released into the extracellular space. The neutrophils can also secrete their agents in large quantities into the extracellular space if the target is too big to be engulfed or if it somehow avoids phagocytosis. This results in an effect not only on the target but also on adjacent tissues.¹⁸

The two major types of granule in neutrophils are a) the azurophil and b) the specific granules. An example of the azurophil granule proteins is MPO. It is often used as a marker protein of neutrophils. However, it is not completely specific for neutrophils. MPO is known to catalyse the interaction of H₂O₂ with Cl⁻ to form hypochlorous acid (HOCl), which is very toxic for a broad range of microorganisms.¹⁸ The specific granule contains a wide variety of different components with largely unknown functions. One of these is the HNL (also called NGAL) and is thought to be more specific for neutrophil activation in bacterial infections and inflammatory disease when neutrophils are involved.¹⁹

Markers of eosinophils

The eosinophil granulocytes are white blood cells that are produced in the bone marrow and enter the blood stream before they migrate into the tissue. Eosinophils are in the migrated mature form mainly found in the gastrointestinal tract. Their main beneficial function is to defend the host against parasitic helminths.²⁰ The activated eosinophils are able to phagocytose particles and are known to have the capacity to present antigen to lymphocytes.²¹ They probably also take part in regulating lymphocytes, but their main killing mechanism is the release of toxic granule proteins and production of oxygen free radicals.²² In the absence of parasites activated eosinophils may cause tissue destruction and inflammation. Activated eosinophils release four cationic proteins: ECP, eosinophil protein X (EPX), eosinophil peroxidase (EPO) and major basic protein (MBP). The first three are considered to be specific for the eosinophils²³ and are therefore often used as a marker for these. Eosinophil accumulation in the gastrointestinal tract is seen in numerous gastrointestinal disorders, including IgE-mediated food allergy, eosinophilic gastroenteritis, inflammatory bowel disease (IBD), eosinophilic esophagitis and gastroesophageal reflux disease.²² Eosinophils represent a small percentage of the total leukocytes seen in IBD but their increased level is thought to be a negative prognostic indicator.²⁴ However, a recent report showing high concentration of activated eosinophils in inactive ulcerative colitis suggests that the eosinophils may also play a role in the repair of injured epithelium.²⁵

Marker of mast cells and basophils

Basophils are normally confined to the circulation and are not found in normal tissue but can infiltrate the site of allergic inflammation.²⁶ Mast cells on

the other hand are resident in tissues throughout the body but are most common at sites that are exposed to the external environment such as skin, airways and intestine. The mast cells share many features with other innate immune effector cells, such as neutrophils and macrophages. Mast cells are endowed with unique effector's capabilities and activation responses that initiate innate immunity to bacteria and are essential to host defence against helminthic parasites.²⁷ They are though mostly known as the major effectors of type I hypersensitivity. But during the last 2-3 decades it has become clear that the mast cell also participates in a number of disease processes in various tissues.²⁸ Mature mast cells contain numerous basophilic granules in its cytoplasm and upon activation they can release mediators to fulfill biological functions. Among the many mediators in mast cells is histamine.²⁹ Histamine has a potent effect on vascular permeability and has been implicated in many of the symptoms of acute allergic reaction. As only mast cells and basophils contain histamine in man, histamine can be used as a marker for these cells.

Nitric oxide as a marker of inflammation

Nitric oxide (NO) was revealed as a biological agent of great importance at the end of the twentieth century. Since then, the field of research of NO has been growing and NO is now regarded to be involved in many systems of the body. NO is a colourless gas with certain instability but in ambient air with 21% oxygen, gaseous NO in biological relevant concentrations (10-10000 ppb), is rather stable.³⁰ In contrast, NO in solution is much less stable³¹ and in vivo the half-life is further reduced by scavenger proteins.³² Nitric oxide is synthesized from L-arginine by the enzyme nitric oxide synthase (NOS), which exists in three isoforms, NOS I (also known as nNOS), NOS II (also known as iNOS) and NOS III (also known as eNOS)³³. NOS II is the major inducible isoform and may become activated as part of immune and inflammatory responses and produces great amounts of NO as long as the enzyme is activated. Increased luminal NO concentration in the small intestine has previously been reported in patients with untreated coeliac disease,³⁴ IBD³⁵ and microscopic colitis.³⁶ Nitric oxide production is thus a part of the inflammatory process but its role in the inflammatory process is uncertain. Some studies indicate a protective role³⁷ and others a harmful role.^{38,39}

Methods to evaluate gut inflammation

Our knowledge about the events in various diseases, including coeliac disease, has long been derived from morphological and immuno-histochemical studies of biopsy and surgical specimens from affected gut areas.⁴⁰⁻⁴² In almost all the diseases of the gut there has been a search for an easier way to evaluate the reaction/inflammation in the gut mucosa. In the last two to three decades new techniques have been evolved and utilized. The rectal dialysis⁴³ and rectal/colonic perfusion techniques⁴⁴ have provided important information about the involvement and activity of various inflammatory cells in inflammatory diseases of the gut,^{15,43,45} but these methods are relatively complicated and are therefore mainly used as research tools.

While the main inflammatory attack on the gut mucosa resulting from exposure to gluten is seen on the proximal intestine, immune cells reactive to gluten antigens appear to be distributed along the mucosa of the entire gastrointestinal tract.^{4,46,47} As the rectum is a site of easy access for investigation, its response to local gluten challenge has been evaluated in multiple mucosal biopsy samples. Such studies have shown that in coeliac disease the rectal mucosa is sensitized to gluten, and they have also elucidated the dynamics of the rectal epithelial lymphocyte and leucocyte responses to gluten.^{4,5,48-52} On the basis of these observations, rectal gluten challenge with mucosal biopsy has been proposed for diagnostic purposes.⁶ However, the histopathological pattern in the biopsy specimens may be difficult to define clearly, and at best only in semi-quantitative terms. Hence, the findings regarding the diagnostic value of rectal gluten challenge have been conflicting.⁵³

The advantage of intestinal and colonic perfusion techniques over morphological and immunohistochemical studies of biopsy specimens is that they allow quantitative evaluation of the release of humoral substances and cells in the diseased gut mucosa.^{44,54} However, for large clinical studies we have recognised the need for a simpler method that is safe, fast and inexpensive, but still reliable for assessing inflammation of the gut mucosa.

Food hypersensitivity

According to the position papers on nomenclature for allergy from the European Academy of Allergy and Clinical Immunology (EAACI) food hypersensitivity includes any adverse reaction resulting from the ingestion of food (Figure 1). It might be the result of food allergy involving an immunologic reaction or it might be a result of a non-allergic food hypersensitivity (formerly called food intolerance).^{55,56} Non-allergic food hypersensitivity is an adverse response caused by some physiological characteristic of the host, including metabolic disorders such as lactase deficiency (lactose intolerance). Food allergy on the other hand is an adverse immunologic reaction

that is due to IgE or non-IgE mediated immune mechanism. Toxic reaction to food containing contaminants (e.g., histamine in tuna fish poisoning) or pharmacological substances within food (e.g., tyramine in aged cheeses) can mimic food hypersensitivity and make the diagnosis more difficult. Food aversion can also cause difficulties in diagnosing food hypersensitivity. It is a psychologically based food reaction that comprises both psychological avoidance, in which the subject avoids food for psychological reasons, and psychological intolerance, which is an unpleasant bodily reaction caused by emotions associated with food, rather than the food itself. Food aversion can not be reproduced in blind or double blind food challenges.^{1,55,57}

Approximately 20% of the industrialized nations populations report an experienced adverse reaction to food.⁵⁸ According to the available tests only about one third of the reactions in children and one tenth of those seen in adults are proven to be allergic reactions. Therefore, confirmed allergy has a prevalence of 6-9% in children and 1-2% in adults.¹

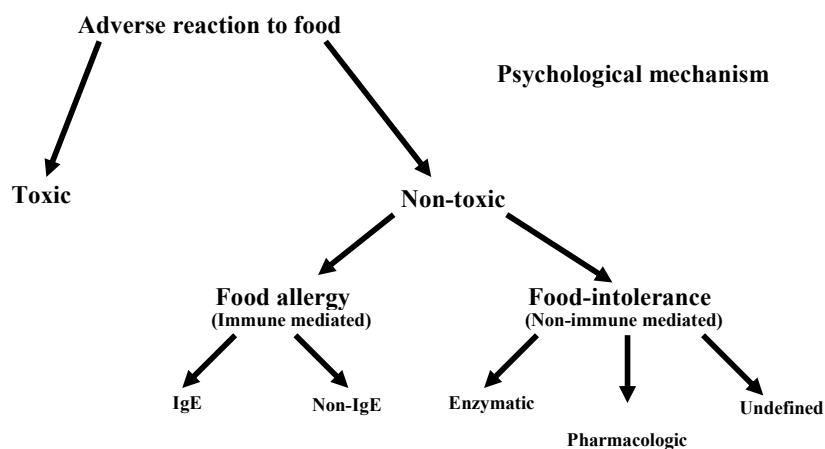


Figure 1. Classification of food hypersensitivity according to EAACI (European Academy of Allergy and Clinical Immunology.)⁵⁵

IgE mediated hypersensitivity (also referred as type I) is probably the best characterized reaction to food. In selected individuals with an immediate IgE mediated food allergy there is a delayed reaction with enhanced cell infiltration of tissues with inflammatory cells and subsequent tissue damage. This kind of delayed reaction and other reactions involving cell infiltration is thought to be the main mechanism in non-IgE allergy. Non-IgE allergy/cell mediated reactions are believed to play the main role in milk and soy protein enteropathies, and in coeliac disease (gluten enteropathy).

Coeliac disease

Coeliac disease was probably first described by Aretaeus, a Greek physician from Cappadocia who practiced in Rome and Alexandria in the beginning of the second century AD. The Greek word “koiliakus” used by Aretaeus originally meant “suffering in the bowels”.⁵⁹ Aretaeus described a problem state, the “coeliac diathesis” which means “large/distended belly”.⁶⁰ In 1888 Samuel Gee, a physician in London, described the “coeliac affection” and recognized that the diet was the main part of the treatment. However, he did not recognize the specific harmful effects of cereals. This was first revealed in 1950 when W.K. Dicke, a Dutch paediatrician, defended his thesis; “Coeliac disease. Investigation of the harmful effects of certain types of cereals on patients suffering from coeliac disease.” The fact that cereals had a role in the pathogenesis of the disease was thereby clarified.⁶¹ The next important advance in the diagnosis of coeliac disease was made 1956 when the per oral jejunal biopsy was introduced.⁶² Since then the histological, clinical and epidemiological facts of the disease have been revealed themselves step by step.

Coeliac disease is a food allergy with permanent intolerance to wheat gluten and related proteins in barley and rye. Most patients seem to tolerate proteins in oats⁶³ but recently a few reports indicate that some coeliac patient might react to these as well.^{64,65} The typical proximal intestinal inflammation leads to destruction and eventual loss of absorptive surface (villous atrophy) and potentially consequences of malabsorption. However the expression of the disease is very complex with multifactorial etiology involving both genetic and environmental factors. The chronic inflammation of coeliac disease has an autoimmune component signified by disease specific autoantibodies to tissue transglutaminase and the disease may be defined as a state of heightened cell-mediated and humoral reactivity to prolamin peptides in genetically predisposed individuals.⁶⁶

Genetics

A high prevalence rate (10% of coeliac disease) among first degree relatives of patients with coeliac disease indicates a strong genetic influence on susceptibility to develop the coeliac disease. The strong genetic influence in coeliac disease is further supported by a high concordance rate in monozygote twins.⁶⁷ During the last decades research has been done to identify the genes involved in the pathogenesis of coeliac disease. Both human leucocyte antigen (HLA) and non HLA genes are known to be important and that these genes shape the immune response to gluten so that immunopathology is produced in the intestine. Available data suggests that susceptibility to develop coeliac disease is primarily associated to two conventional peptide-presenting HLA DQ molecules on antigen presenting cells: i.e. peptides named DQ2 and DQ8. About 90-95% of the patients with coeliac disease are

HLA DQ2 and the remaining are HLA DQ8.⁶⁶ The DQ2 and DQ8 molecules bind gluten peptides that after specific deamination become good peptide ligands for DQ2 and DQ8. However, DQ2 is demonstrated in 25-30% of healthy controls, which indicates that additional factors determine the development of coeliac disease.⁶⁸

Gluten, gliadin and related protein

The proteins collectively known as gluten are the proteins known to be left when starch has been removed from the grain. Gliadin is the alcohol soluble protein fraction of gluten and is the part that contains the most harmful proteins to those susceptible to coeliac disease.⁶⁹ In rye the toxic alcohol soluble storage prolamins are called secalin and in barley hordein. In oats the prolamins (avenins) are thought to be harmless but as mentioned before this might not be the case for all coeliac patients and perhaps the presumptive harmlessness reflects the reduced amount of prolamins in oats, which represent only 5-10% of the storage proteins in oat compared with 50 % in wheat.⁷⁰ Another explanation could be that oat is not as closely related to wheat as rye and barley and therefore might be less toxic.⁷¹ The relations of different cereals are illustrated in figure 2.

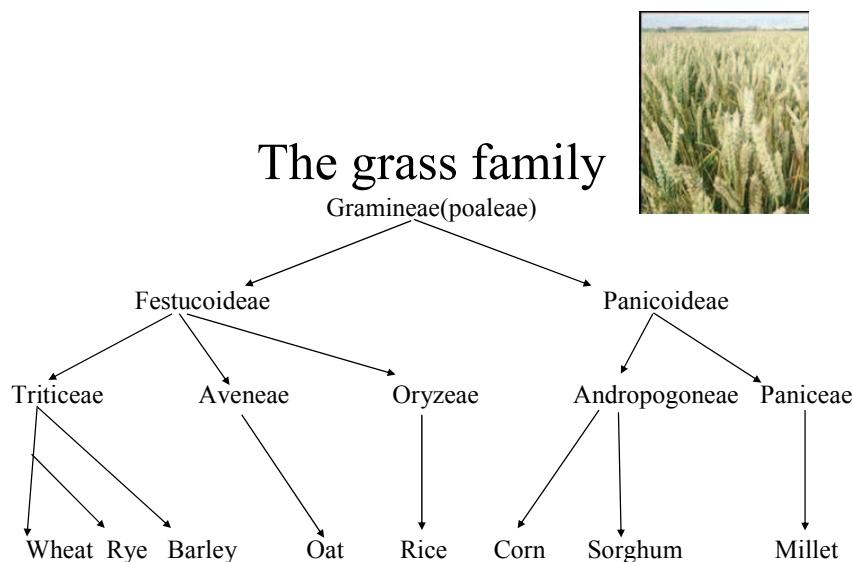


Figure 2. Illustration of the relationship of different cereals^{71,72}

Histopathology and mechanism in coeliac disease

As mentioned above coeliac disease is an enteropathy that develops in genetically susceptible individuals upon exposure to dietary gluten. The classical lesion involves villous atrophy, crypt cell hyperplasia, lymphocytic infiltration of the epithelium and increased density of various leukocytes in the lamina propria.⁷³ Marsh was the first to suggest a sequence of progression in the coeliac lesion.⁷⁴ The initial event observed (stage 1) is an increase in intraepithelial lymphocytes, followed by infiltration of the lamina propria with lymphocytes. This is followed by a crypt hyperplasia (stage 2) preceding the well known villous atrophy of the intestinal mucosa in coeliac disease (stage 3). But the mechanism by which gluten and the inflammatory cells exert their damaging effect on the mucosa is still unclear. Even the role of autoantibodies has been questioned.⁶⁸ With today's knowledge a suggestion of patho-mechanisms seen in the coeliac reaction is shown in Fig 3. Wheat gluten or similar prolamins are stable to digestion due to their content of proline⁷⁵ and can therefore be presented to the immune system of the gut when the permeability of the mucosa is increased. This is seen in small intestinal infections or after chemical injury.⁷⁶ Such injury also results in a local release of the intracellular enzyme, tissue transglutaminase (tTG). This enzyme comes mainly from fibroblasts and endothelial cells⁷⁷ and has a key role in the maintenance of tissue stability (cross-links several matrix proteins and thus stabilizes the scaffold of connective tissues).⁷⁸ Transglutaminase crosslinks and deamidates gluten and thus enhances the binding of gliadin peptides to the HLA DQ2 and 8 molecules on leucocytes. This enhanced attachment seems to intensify T-cell activation and therefore a greater T-cell response to gluten in subjects with this susceptibility.⁶⁶ Professional antigen presenting cells (B-cells, macrophages and dendritic cells) present gliadin, deaminated gliadin and gliadin-tTG complexes on the HLA molecules to gliadin specific T-cells. These T-cells stimulate B cells to produce antibodies to gliadin, gliadin-tTG complex and to the tTG itself. The autoantibodies to tTG are very specific for coeliac disease and have been used as a diagnostic tool,⁷⁹ but their role in the mechanism of mucosal damage is still unclear.

Activated T-cells also stimulate production of cytokines which leads to damage of the mucosa and villi, resulting in enteritis with an increase in inflammatory cells, fibroblasts and endothelial cells. These cells then release and activate tTG and with the presence of gliadin protein leads to a new activation and a vicious circle ensues, as long as there is an intake of gliadine or similar prolamines. A pathophysiological mechanism in coeliac disease is thus mainly dependent on an activation of T and B lymphocytes, an activation of the adaptive immune system. However, findings of recent studies have shown that a fragment of gliadin has the ability to induce an activation of the innate immune response as well. Such activation seems to dictate the type and intensity of the immune adaptive response controlled by pathogenic

CD4+ lamina propria cells.⁸⁰ This is congruent with the theories of a danger signal. The theory is that the mere recognition of the HLA bound-antigen is not sufficient to induce a protective T-cell immune response. T cells have to recognize antigen in the presence of an activated innate immune system.⁸¹ In coeliac disease that would be an infection or a chemical reaction to a substance

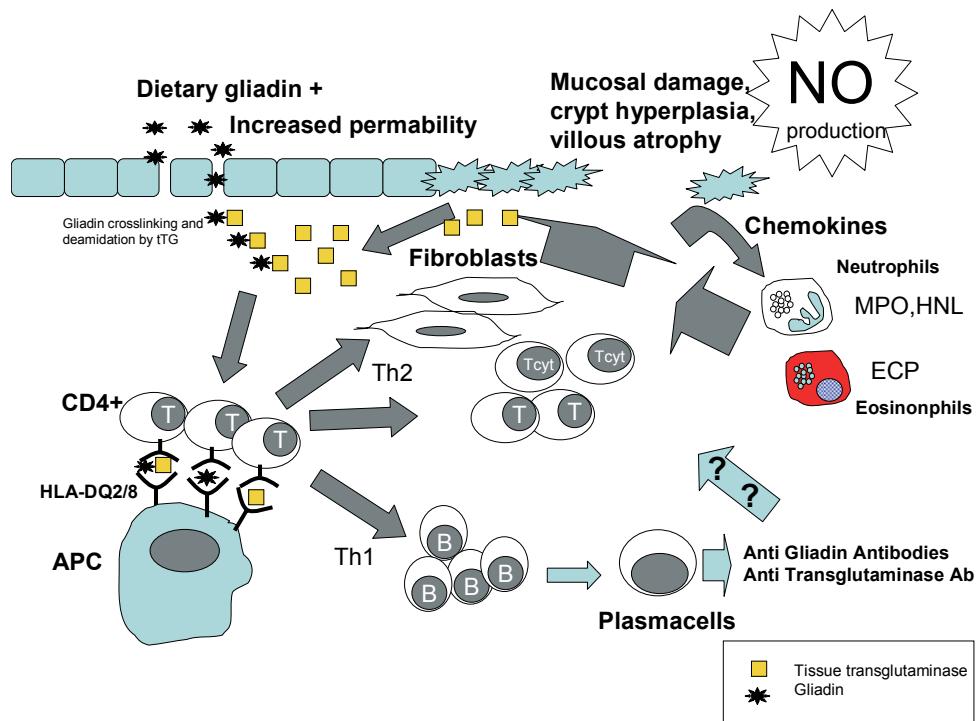


Figure 3. A suggestion/illustration of the pathophysiological mechanisms in coeliac disease. Gliadin reaches the lamina propria, especially when the permeability is increased after infection, chemical or mechanical stress. Tissue transglutaminase is then released and activated. Crosslinking and deamidation of gluten peptides creates potent immunostimulatory epitopes presented via HLA-DQ2 or DQ8 on antigen presenting cells (APC). These cells activate CD4+ T-lymphocytes (T) which leads to either an antibody production (activation of B-cells (B)) or to an inflammation and remodelling of the mucosa. Chemokines from the activated lymphocyte also activate neutrophils and eosinophils who take part in the inflammatory reaction. Nitric oxide (NO) is produced in the gut mucosa but its role is not known.

Epidemiology

The “coeliac iceberg” is a phenomenon or a model illustrating the epidemiological understanding of this disease and its multi-factorial etiology men-

tioned above (Figure 4.). The tip of the iceberg (the visible part) is patients who are diagnosed because of their symptoms and sometimes called “active disease”. Beneath the surface there are individuals who are diagnosed in population screening studies and are asymptomatic or have very mild symptoms, also called “silent disease”. Beneath this group in the iceberg there are individuals with susceptibility to develop coeliac disease, the group of patients with “latent disease”. On the bottom, but perhaps the largest group are those people having the heritage but who never will develop the disease. The environmental factors that influence the change from having the susceptibility to silent or active disease are not known. The disease is primarily a disease in Caucasians.⁷³ and until some years ago the disease was thought to be mainly a disease seen in Europe.⁸² Reported prevalence of disease with overt symptoms varies enormously in the population of Europe and USA. Especially high prevalence has been reported from countries such as Sweden^{83,84}, Ireland⁸⁵ and Italy.⁸⁶ New studies assessing the prevalence by biopsy examination of individuals identified by antibody screening (tTG and EmA) have however demonstrated surprisingly similar prevalence rates; about 1:120-1:400 throughout Europe, Asia⁵⁹ and America.⁸⁷ In one country after another a “coeliac iceberg” is found and health care professionals throughout the world are becoming increasingly aware of the disease.

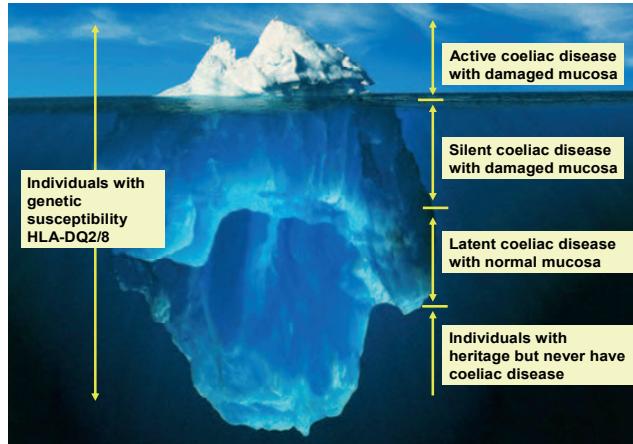


Figure 4. The “iceberg” is an illustration of the different clinical manifestations of coeliac disease and its genetic predisposition. The iceberg model explains different epidemiological data seen in the world. The patients diagnosed because of symptoms being the iceberg above sea level and defined as active disease. Below the water level are the patients with genetic susceptibility that are diagnosed in screening of relatives or in studies. These patients are referred to as having silent disease. The lowest part of the iceberg consists of the patients with latent coeliac disease that will later present with a silent or active coeliac disease or individuals that never will have the disease but have the genetic susceptibility.

Antibodies

Because of the invasive nature of the diagnostic procedure and the large spectrum of non-specific symptoms (from none to an extensive malabsorption) there has been a need for a non-invasive screening marker or a test that is less demanding for the patients and the investigator. Various serological markers have been studied and proposed for this purpose. First to be discovered and proposed for use in diagnosing coeliac disease was the presence of anti-gliadin antibodies (AGA)⁸⁸⁻⁹⁰ but later studies showed low sensitivity and specificity^{91-93, 94}. In 1983 Chorzelski et al described anti endomysium antibodies (EmA).⁹⁵ EmA in studies has shown a relatively good sensitivity and very good specificity.⁹⁶⁻⁹⁹ Tissue transglutaminase (tTG) as EmA's autoantigen was discovered in 1997.¹⁰⁰ Auto-antibodies to tTG of IgA and G types have been found with similar sensitivity and specificity as EmA.⁷⁹ EmA and tTG antibodies offer a high sensitivity and specificity but the sensitivity of these tests appears to be lower than reported when milder histologic grades are used to define CD (below 90%).¹⁰¹ Also patients with coeliac disease and IgA deficiency will be missed using these IgA based tests. The nearly perfect negative predictive value reported of these tests would therefore drop. Furthermore, the positive predictive value (PPV) of the test is likely to be lower than reported when the tests are applied to low-prevalence populations as many of the studies on sensitivity and specificity are made in high prevalence population. This is important because the proportion of patients who would undergo unnecessary further testing will rise if PPV falls. Therefore, confirmatory biopsy still plays an important role in the diagnosis of coeliac disease and serological tests have not become the solution to the practical problem of the diagnostic procedure as once hoped. Serological tests do support the diagnosis and have given us a practical tool for screening and for the selection of asymptomatic relatives requiring further investigation with duodenal biopsies.

Diagnostic criteria of coeliac disease

Several studies have shown that there is a significant delay (often many years) between initial onset of symptoms and the diagnosis.¹⁰² The single most important step in diagnosing coeliac disease is to first consider the disorder by recognizing its great variation in clinical presentation. Coeliac disease is characterized by a gluten-induced inflammatory reaction in genetically predisposed subjects. After continuous contact with gluten, crypt hypertrophy and villous atrophy develop. The jejunal histological changes remain the cornerstone for the diagnosis of coeliac disease. However, in some patients the biopsy specimens obtained from the jejunum show only minimal changes (Marsh type 1) making the diagnosis of coeliac disease difficult and reserved to experienced pathologists. Thus there is a wide spectrum of clin-

cal features, laboratory and histopathologic findings. We need a combination of these to help us in diagnosing coeliac disease.

The European Society of Pediatric Gastroenterology and Nutrition (ESPGAN) proposed the first criteria in 1969, this was then confirmed and refined slightly in 1978.¹⁰³ The criteria proposed comprised three small bowel biopsies. One biopsy was taken on gluten diet showing classical histological damage in small bowel mucosa. A second biopsy taken after gluten free diet was supposed to confirm a healing of the mucosa and the third and last biopsy was taken after challenge with gluten confirming it as the cause of the findings.

In 1990 ESPGAN revised the criteria.² The requirements then included the characteristic histological changes of the mucosa before diet with disappearance of symptoms after gluten elimination. New biopsy or gluten challenge is not mandatory but should be used in cases where there are doubts about the diagnosis or when patients do not have any symptoms to improve (silent coeliac disease). Serological tests with increased AGA or EmA before gluten free diet which then decreased after diet, were considered supportive of the diagnosis. These criteria are still used with some small adjustments (using tTGA instead of EmA).

Rectal provocation

As described above the diagnosis of coeliac disease still relies on the histological changes in intestinal mucosa but studies of intestinal mucosal inflammation are limited by the relative inaccessibility of most of the small intestine. While the main inflammatory attack on the gut mucosa resulting from exposure to gluten is seen in the proximal intestine, immune cells reactive to gluten antigens appear to be distributed along the mucosa of the entire gastrointestinal tract.^{4,46,47} As the rectum is a site of easy access for investigation, its response to local gluten challenge has been evaluated in multiple mucosal samples. Such studies have shown that in coeliac disease the rectal mucosa is sensitized to gluten, and they have also elucidated the dynamics of the rectal epithelial lymphocyte and leucocyte responses to gluten.^{4,5,48-52} On the basis of these observations, rectal gluten challenge with mucosal biopsy has been proposed as a diagnostic tool.⁶ However, the histopathological pattern in the biopsy specimens may be difficult to define clearly, and at best only in semi-quantitative terms. Hence the findings regarding the diagnostic value of rectal gluten challenge have been conflicting⁵³ and very few clinics have adapted this technique in their diagnostic routine.

Clinical feature

The clinical manifestations in coeliac disease are highly variable. It may present at any age and can involve any of the organ systems. On the extreme end of the clinical spectrum is the patient with classical symptoms, extremely malnourished with diarrhoea and weight loss, and at the other end is

the symptom-free patient that is diagnosed by accident in a health screening program. Table 1 illustrates the most common symptoms in coeliac disease. It is widely accepted to divide coeliac disease into classic (diarrhoea predominant) and the silent type of coeliac disease. The silent group includes truly asymptomatic patients and those with atypical presentation (like osteoporosis) and presenting only with complications or consequences of the inflammation and villous atrophy. The symptoms in coeliac disease can be attributed to three main reasons. First there are direct consequences of inflammation and architectural changes in gut mucosa. Examples of this are symptoms of diarrhoea and abdominal pain causing IBS-like symptoms.^{104,105} Secondly symptoms can be explained by secondary consequences of the morphological changes in the small intestine causing malabsorption of vitamins and nutrients. Examples of these are the most common symptoms in coeliac disease, tiredness and lethargy that partly can be caused and explained by iron deficiency or folate deficiency often seen in the coeliac patients.¹⁰⁶ Other symptoms which are more infrequent and harder to relate to the GI tract are the neurological symptoms, like peripheral neuropathy¹⁰⁷, problem with concentration, ataxia,¹⁰⁸ depression etc that might be caused by deficiencies of B-vitamins. The reason for symptoms in extra intestinal organs in coeliac disease may be ascribed to toxic or immunological reactions.

Table 1. Some of the clinical manifestations of coeliac disease¹⁰⁹

Gastrointestinal	Non-gastrointestinal
Chronic diarrhoea	Anaemia
Steatorrhoea	Fatigue
Weight loss	Folate and /or iron deficiency
Failure to thrive	Infertility
Vomiting	Dementia
Dyspepsia	Neuropathy
Elevated transaminases	Ataxia
Recurrent pancreatitis	Tetany
Constipation	Osteoporosis
Heme positive stools	Arthralgia
Bloating	Osteomalacia
Abdominal pain	Dermatitis herpetiformis
Enteropathy-associated T-cell lymphoma	Seizures
Duodenal obstruction	Depressions
	Enamel defects

Associated diseases

To complicate the clinical manifestation of the disease there are many associated diseases or conditions to coeliac disease. Among patients with coeliac disease 30% may have at least one associated autoimmune disease;^{110,111} this compares with 3% in the general population.¹¹² Examples of these autoimmune conditions are type 1 diabetes, thyroiditis and Sjögren's syndrome¹¹³.

Other associated diseases are IgA nephropathy, genetic disorders like Down's syndrome,¹¹⁴ intestinal lymphoma, adenocarcinoma of the small intestine¹¹⁵ and neurological diseases like cerebellar ataxia.¹¹⁶ Associated gastrointestinal diseases such as the microscopic colitis and patients with persistent or recurrent diarrhoea should have colonic biopsies taken to rule out coexisting collagen colitis.¹¹⁷

Management of coeliac disease and persistent or recurring symptoms

The only true treatment of coeliac disease is a gluten free diet for life. To achieve full compliance complete information and consultation with a skilled dietitian is needed. Many patients have problems with identifying gluten containing foods. In the simplest terms a gluten free diet is defined as a diet that excludes wheat, rye, barley and in some cases oat. This is not as easy as it sounds. Ingredients are not always declared properly and trace amounts and contamination can be a problem in some cases. But even with good knowledge and technical equipment the strict definition of gluten free diet remains controversial around the world. The reason for this is a lack of accurate laboratory methods to detect gluten in food and lack of scientific evidence for what constitutes a safe amount of gluten ingestion. According to the WHO/FAO Codex Alimentarius, gluten free diet is food containing up to 0.3% of protein from gluten-containing grains and probably this is adequate for most patients.¹¹⁸ Symptoms have though been reported by some coeliac patients after prolonged consumption of small amounts of gliadin^{118,119} and there is a variation in sensitivity to gluten in patients.¹²⁰ Resolution of symptoms may take up to 3-6 months and complete healing of the mucosa even longer, especially in the elderly.

In spite of strict gluten-free diet a considerable fraction of adult patients with coeliac disease do not normalise the small intestine mucosa.¹²¹ The most common reason for this is as mentioned above compliance problems in adhering to lifelong strict gluten-free diet. But there are coeliac patients who, in spite of good compliance and a strict gluten free diet, still have abnormalities in histopathologic appearances on a new biopsy.¹²² In these patients it is very important to rule out alternative causes for the enteropathy such as infectious diseases (tropical sprue or giardiasis etc), toxic damage of mucosa by drugs (NSAID) or alcohol etc. Non-gluten food intolerance should also be considered in at least children.^{121,123} Only few studies have focused on non-gluten dietary intolerance in celiac patients and much of what is written and stated is based on clinical experience and case reports. Many physicians may though recommend elimination of cow's milk protein or soy protein when their patients with coeliac disease do not recover on a strict gluten free diet.^{124,125}

Milk protein allergies/intolerance

Cow's milk (CM) has a high nutritional value and is one of the most commonly consumed foods worldwide. Nevertheless, adverse reactions to CM are frequently reported and are mostly attributed to lactase deficiency or allergy to CM proteins.¹²⁶ As with food allergy in general (see above), CM allergy may be divided into IgE-mediated and non-IgE-mediated food allergy, the latter mainly type III and IV immune reactions. Most often allergic reactions to CM proteins have been attributed to an IgE-mediated mechanism, at least in childhood.¹ A typical clinical history, a positive skin prick test and/or increased IgE antibody levels to milk proteins may support the diagnosis.¹²⁷ Non-IgE food allergies are more difficult to evaluate, both clinically and in the laboratory and require procedures with food elimination and food challenges.^{1,128} Coeliac disease is the best characterized non-IgE food allergy with a food protein-induced enteropathy. Cow's milk protein may also induce a non-IgE enteropathy, which is considered to be a transient condition in early childhood but may persist or manifest itself in older children.¹²⁹ The histopathological features of the small intestine in coeliac disease are often prominent, while the histological inflammatory findings in CM protein-sensitive enteropathy are more discrete, with normal villous architecture.^{129,130} Very little is known about non-IgE food allergy in adults.

Aims of present investigation

The aims of these studies were to develop and evaluate a new technique to characterize rectal local inflammatory process after rectal food challenge in patients with coeliac disease.

Specific aims were:

- to develop and evaluate a new technique, the mucosal patch technique, from a methodological point of view for studying humoral inflammatory mediators from various cell populations in rectal mucosa.
- to elucidate the dynamics of the rectal inflammatory response to rectal gluten challenge in patients with coeliac disease by using the established perfusion technique combined with the new mucosal patch technique to measure inflammatory mediators released by activated neutrophils, eosinophils and mast cells/basophils.
- to elucidate the dynamics of NO production induced by rectal gluten challenge and the relation between NO production and mucosal granulocyte activation measured by local release of cell specific substances.
- to study the inflammatory response to rectal milk challenge in controls and patients with coeliac disease.

Ethics

All participants of the study gave informed consent. The Ethics Committee of the Medical Faculty, Uppsala University, approved the study.

Patients and Methods

All patients in the studies were recruited at the Section of Gastroenterology at the University Hospital of Uppsala.

Control subjects

Totally twenty-three healthy adult subjects served as controls in these studies. They had normal blood test values (no signs of inflammation, malabsorption or disease) and no gastrointestinal symptoms, and they had had no history of illness the last year. None of the controls had a rise in IgA antibodies to gliadin or endomysium or IgE antibodies to wheat or milk. One had a border value for IgA antibodies to tissue-transglutaminase but no other signs of coeliac disease and normal duodenal biopsy results.

In study I

Eighty-one subjects and patients, 40 men and 41 women, were recruited. Measurements with the mucosal patch technique were performed in healthy controls (n=16) and patients with active (n=19) and inactive ulcerative colitis (UC, n=8), collagen colitis (CC, n=12), coeliac disease (n=13) and irritable bowel syndrome (IBS, n=13)

The diagnoses of IBD were based on clinical and endoscopic criteria¹³¹ and x-ray of the small intestine. For grading the activity of UC, a four-graded endoscopic scale was used, where a score of 1 represents normal mucosa and scores 2-4 imply increasing degrees of visible inflammation.^{131,132} Patients were judged to have inactive UC when normal macroscopic findings at endoscopy were combined with normal blood tests and normal bowel movements. Active UC was diagnosed on the basis of the clinical symptoms and when endoscopic score was 2-4. Patients with collagen colitis were all diagnosed by clinical symptoms and colonic biopsies. The IBS patients fulfilled the ROME II criteria.¹³³ The patients with coeliac disease were all diagnosed on the basis of two duodenal biopsies, showing regression or normalisation of the duodenal mucosa after a gluten-free diet.

In study II

Nineteen adult patients with coeliac disease were studied and twenty healthy adult subjects served as controls. In order to study the early and late inflammatory reaction to gluten we used two different devices, rectal perfusion and mucosal patch technique. The early reaction was studied by continuous per-

fusion of the rectum. In order to make repeated measurements possible during the late challenge period, we used the mucosal patch technique, which is much better tolerated.

Fourteen subjects (9 patients (7 female/2 male with mean age 45(range 20-63)) and 5 controls (4 female/1 male with mean age 37(28-52))) were studied with rectal perfusion, before and 3 hours after rectal gluten challenge. Five of the patients had recently been diagnosed with coeliac disease and were on a normal diet at the time of the investigation. Twenty-five other subjects (10 patients (5 female/5 male with mean age of 50(range 25-68) and 15 controls (5 female/10 male with mean age 27(19-51))) were studied with the rectal mucosal patch technique. Ten patients with coeliac disease, all on a gluten-free diet and with normal results of wide blood test screening, underwent rectal mucosal patch measurements before and 5, 15, 24 and 48 hours after rectal gluten provocation. Fifteen healthy subjects served as controls; and rectal mucosal patch measurements were performed before and 15 hours after gluten challenge.

In study III

The study comprised 13 patients with coeliac disease (6 males, 7 females), mean age 48 years, range 25-68 and 18 healthy control subjects (13 males, 5 females), mean age 32, range 19-58. All patients had been on a gluten-free diet for an average of 12 years, range 6-22. After gluten free diet small intestinal biopsy results were normal (Marsh 0) in 9/13 patients; 4/13 had a partial remission (Marsh 1). At the time of the present investigation all patients had IgA tissue transglutaminase and IgG/IgA gliadin antibodies within the normal range and no detectable IgA endomysial antibodies. All patients with coeliac disease had normal blood test results at the time of the investigation.

All subjects underwent a rectal provocation with gluten, and mucosal, blood and luminal nitric oxide measurements were performed before and after challenge. The mucosal patch technique was used for mucosal evaluation and for rectal luminal NO measurement we collected air from the balloon on the instrument used for the mucosal patch technique. Initially, in ten of the CD patients measurements were made before and 5, 24 and 48 hours after rectal gluten challenge. These results indicated the need for 15 hours measurements, and a further gluten challenge was therefore performed in all patients and measurements were carried out at 15 hours in addition to the other times mentioned. Two patients also underwent a 10-hour test but as the NO peak occurred at 15 hours we decided not to continue with tests at 10 hours. The controls were tested before and 15 hours after challenge. Ten of the patients and seven of the controls underwent a second test with corn gluten for evaluation of the specificity of the reaction.

In study IV

Twenty adult patients (six men) with CD and 15 adult healthy control subjects (ten men) were included. The mean age of the celiac patients was 49 years (range 25-68) and that of the controls was 34 years (19-58). Prior to dietary treatment 17 of the CD patients had total/subtotal villous atrophy and three had partial villous atrophy. At the time of the present investigation all patients had been on a gluten-free diet for more than 2 years (range 2-22). On gluten free diet, small bowel biopsy results became normal in 11/20 patients and the other nine had partial remission. At the time of the present study, all patients had serum IgA tissue transglutaminase (tTG) and IgG/IgA gliadin antibodies within the normal range and no detectable serum IgA endomysial antibodies. Serum IgE-antibodies to cow's milk (CM) or wheat proteins and IgA and IgG antibodies to casein and α -lactalbumin were measured in accordance with the manufacturer's instructions (Pharmacia Diagnostics AB, Uppsala, Sweden).

All subjects underwent rectal challenge with gluten and CM, and mucosal measurements were performed before and 15 hours after challenge.

Rectal challenges (Study II-IV)

All the participants in studies II-IV were challenged with wheat gluten 6.2-6.5 g (Crude wheat gluten, Sigma Chemical Co., St. Louis, Missouri). In study III, a rectal challenge was performed with corn gluten 6.2-6.5 g, (Corn gluten meal, Sigma Chemical Co., St Louis, Missouri) suspended in 25 ml 0.9% NaCl solution. In study IV we challenged patients and controls with dried milk powder 6.2-6.5 g (Semper AB, Stockholm, Sweden) suspended in 25 ml of 0.9% NaCl solution. Six of the 10 patients who had a mucosal inflammatory reaction after cow's milk challenge were challenged with specific milk proteins in amounts proportional to their concentrations in 6.5 g dried CM powder. Thus, six patients were challenged with 1.9 g casein from CM with the normal milk proportions of alpha and beta casein milk protein (Sigma Chemical Co., St. Louis, Missouri). Five of these patients were also challenged with 0.2 g of α -lactalbumin (Sigma Chemical Co.).

Mucosal Patch Technique (Study I-IV)

The instrument of mucosal patch technique used is a plastic catheter with a silicon balloon at the end of the catheter with two or three patches attached to the balloon. The patches are made of highly absorptive cellulose material (Pharmacia Diagnostics AB, Uppsala, Sweden). When the instrument is positioned in the rectum, the balloon is inflated with air (60-80 ml), allowing the patches to be in contact with the mucosa.

If the patient had a strong feeling of distension or pain that did not disappear after 2-3 minutes, the volume of air was reduced by 5 ml at a time until tolerance was reached. A minimum of 50 ml of air was left in the balloon to ensure that the patches remained adherent to the mucosa. The balloon was kept inflated for 20 minutes in all subjects. After 20 minutes the balloon was deflated and the catheter removed. The patches were cut off and immediately put into 2 ml of 0.3% CTAB (N-Cetyl-N,N,N-trimethyl ammonium bromide [E Merck, Darmstadt, Germany]) solution to extract the contents. At each measurement a blood sample was taken. The aim was to evaluate systemic inflammatory activity and for comparison to the measurements of the patches. Any discomfort, reactions and other symptoms during the test procedure were recorded as was the presence of blood or faeces on the patches.

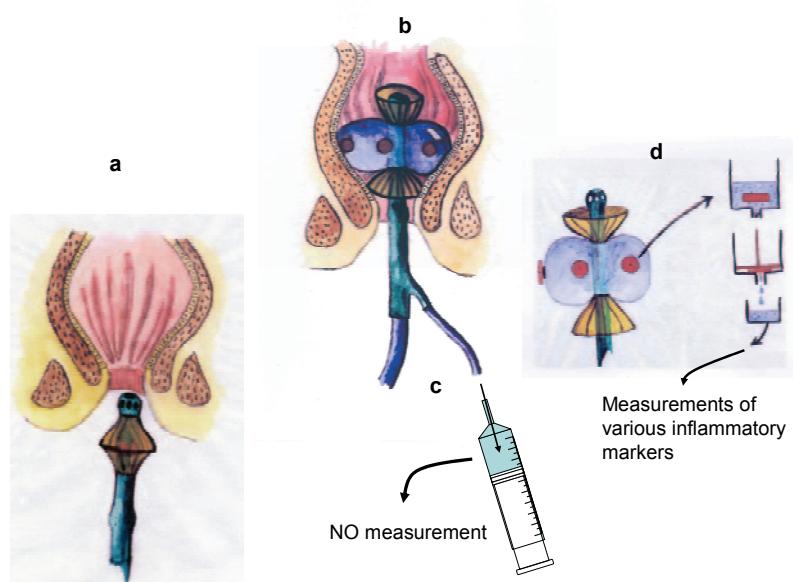


Figure 5. A schematic drawing of the instrument of the mucosal patch technique used for measurements of the inflammatory condition of the rectal mucosa. Fig. 5a illustrates the instrument with non-inflated balloon and with the patches protected by shields. Fig. 5b shows the instrument in position with the inflated balloon in the rectal ampulla and with the patches in contact with the mucosa. Fig 5c illustrates when the balloon is deflated and the air is collected in a glass syringe for analysis of NO. Fig 5d illustrates when the patches are cut off after removal of the catheter from the rectum and immediately placed in 2 ml of 0.3% CTAB (N-cetyl-N,N,N-trimethyl ammonium bromide) to extract the contents for later analysis of inflammatory markers.

Each patch was collected and then analysed separately which meant three sets of measurements for each test procedure. The patches were kept in the 0.3% CTAB solution for extraction for 1 hour. After completion of this step, the extraction solution was squeezed out of the patches, centrifuged and then

frozen at -70 °C until analysed. Blood samples were centrifuged and the serum frozen at -70 °C until analysed. See figure 5.

Rectal biopsies

In study I rectal biopsies were taken in close connection (on average ± 1.8 days) to the present investigation in nine patients with active UC, five with inactive UC and in ten patients with IBS. In order to elucidate the link between the measured mediators and rectal histology, a pathologist examined these biopsies without knowledge of the endoscopic activity score and the results of the mediator measurements. The activity of inflammation was assessed using a slightly modified scoring system that has been validated for ulcerative colitis.¹³⁴ Shortly, seven parameters were judged and scored separately whereas score "0" corresponds to no inflammatory activity (normal histology) and score "3" to the maximal inflammatory activity or destruction observed (Table 2, page 38).¹³⁴

Colorectal perfusion (Study II)

The colorectal perfusion technique has been described in detail elsewhere.⁸ Briefly, a five-channel polyvinyl chloride tube, inner diameter 10 mm and outer diameter 16 mm, with a total length of 38 cm, was used. Three inflatable latex balloons were attached to the tube, creating an 8 cm-long, and closed perfusion segment in the rectum. The channels were used either to inflate the balloons, to perfuse the segment or to administer a dye marker at the tip of the tube.

Two rectal perfusions were performed in each participant on the same day, one basal perfusion and one post-challenge perfusion three hours after rectal instillation of gluten. The tube was introduced into the rectum using the endoscope as a guide. When the desired position was reached, the endoscope was withdrawn and the balloons were inflated with air. Finally, the position of the tube was checked fluoroscopically. Using the inflow channel, the rectal segment was continuously perfused at a rate of 3 ml/min using a syringe pump. The pre-challenge perfusion was terminated after 80 min. The tube was withdrawn after deflation of the balloons. A gluten enema was then given as described above. Two hours after the instillation of the gluten enema, the patient received a 500 ml enema of perfusion buffer to rinse the rectum and the perfusion tube was inserted again. The second rectal perfusion started 180 min after the gluten challenge and was continued for another 180 min. Samples were collected on ice at 20-min intervals. After centrifugation of the samples, the supernatants were immediately frozen in 0.5 ml aliquots at -70°C until they were analysed in duplicate for ECP, MPO and histamine by radioimmunoassay (Pharmacia Diagnostics, Uppsala, Sweden).

The perfusion fluid samples were analysed in sequence to avoid inter-day variation. The variability was less than 10% for all methods.

Analyses of inflammatory mediators (Study I-IV)

Duplicate samples were analysed. HNL was measured with a radioimmunoassay(RIA) previously described.¹³⁵ MPO,ECP and Histamine was measured with a RIA, Pharmacia Diagnostics AB, Uppsala, Sweden, in most of the measurements (paper I-IV). In the end of the studies the RIA's for MPO and ECP from Pharmacia diagnostics were not available and we measured MPO and ECP with an enzyme-linked immunosorbent assay ELISA from Pharmacia diagnostics AB, Uppsala, Sweden. The ELISA and RIA results correlated well ($r=0.997$ for MPO and $r=0.989$ for ECP). In paper IV the results of the ELISA are corrected to the level of the RIA results to give comparable results.

NO measurement (Study III and IV)

NO was measured with a chemiluminescence NO analyser (model Sievers NOA 280; Ionics Instrument Business Group, Boulder, Colorado, USA.). The system was calibrated with a mixture of NO in N₂ (AGA Gas AB, Lidingö, Sweden) with an NO concentration of 500 parts per billion (ppb). The calibration was tested every morning and zero was set before each measurement. Air samples were collected with three glass syringes from the cuff of the rectal catheter described above. Each sample was measured separately. The air was injected into the NO analyser and the peak level was monitored. The single measure of each subject represents the highest value obtained in the collected air samples to avoid false low NO measurement due to syringe leakage. An extraction test was performed to see how much NO could be recovered with our instrument and it was found that 80% was recovered, a result in accordance with earlier reports¹³⁶

Statistical methods

The geometric mean value of the measurements of three patches from each individual was calculated. Mean and standard error of the mean (SEM) of patient and control groups are presented in the figures, tables and text when not stated otherwise.

Mann Whitney U, Sign test, Friedman Anova and Spearman's rank correlation tests were used for statistical analyses. The statistical calculations were performed on a personal computer using the statistical package Statistica (Statsoft Inc., Tulsa, Oklahoma, USA).

Results

Evaluation of a new technique to characterize a rectal local inflammatory process, the “Mucosal Patch Technique” (Study nr I)

Compliance, tolerance and safety

The test procedure was very well tolerated by the patients. Twenty-two controls and patients underwent total colonoscopy 0.5-3 hours after completion of the rectal test, and no signs of trauma to the mucosa were observed. When testing influence of subject preparation the type of gut preparation (hypotonic sorbitol rectal enema or oral laxative isotonic solution) had no significant influence on the recovered amounts of MPO or ECP in tested patients with normal endoscopic findings (n=26) and healthy controls (n=16).

Intraindividual variation

In 11 patients with coeliac disease and 3 healthy controls, two separate measurements were performed on two occasions with an interval of at least one week. The mean MPO concentration was $43.2 \mu\text{g/L} \pm 13.1$ at the first test and $54.6 \mu\text{g/L} \pm 14.8$ at the second one ($p=0.6$). The corresponding ECP values were $29.2 \mu\text{g/L} \pm 7.0$ and $30.5 \mu\text{g/L} \pm 7.4$ ($p=0.79$) respectively.

Time dependency of absorption

Fifteen patients were tested with the instrument positioned in the rectum for various lengths of time. The subjects were divided into three groups on the basis of the degree of inflammatory activity, defined by rectal recovery of the granulocyte markers: those with high (n=5), medium (n=5) and low levels (n=5) of inflammatory markers. The time dependency was not critical for the groups with high and low inflammatory activity, but in the medium group there was a tendency to higher values with time; this difference was not statistically significant ($p=0.11$).

Patient and control measurements

Mucosal measurements

Figure 6 shows the mean recovered mucosal amounts of MPO, HNL and ECP in the patient and control groups. An apparent covariation was seen between MPO and HNL ($r=0.94$ for all individuals). Significantly higher concentrations of all three variables were observed in patients with active UC compared to those with UC in remission. A positive correlation (Spearman $R=0.81$, $p<0.001$) was found between endoscopic disease activity score and MPO concentration in UC patients. The endoscopic findings were also significantly correlated to the HNL (Spearman $R= 0.78$, $p< 0.001$) and ECP concentrations (Spearman $R= 0.61$, $p <0.001$). The other patient groups all had significantly lower concentrations of the measured substances compared to patients with active UC.

Patients with coeliac disease, collagen colitis and IBS had significantly higher levels of MPO and HNL than healthy controls.(figure 6)

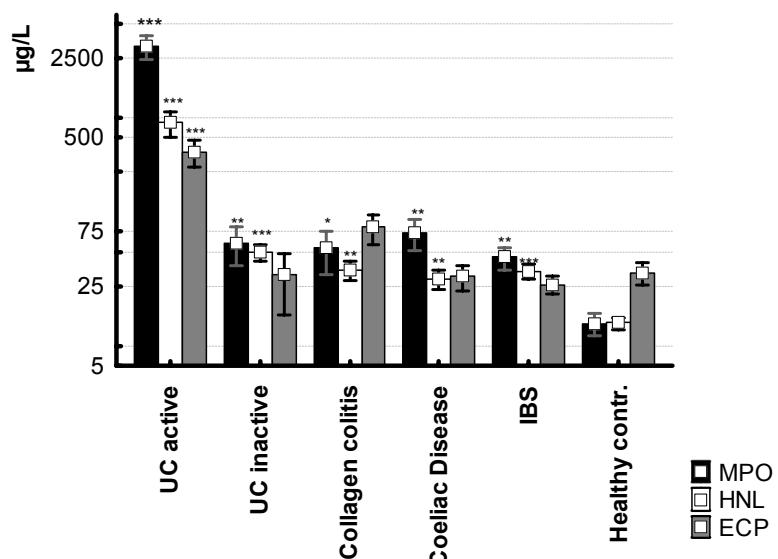


Figure 6. Rectal mucosal concentrations ($\mu\text{g}/\text{L}$, Mean \pm SE) of MPO, HNL and ECP in patients with inflammatory bowel disease, coeliac disease and IBS, and in healthy controls. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ compared to healthy controls.

Mucosal measurements and histological assessment of inflammation.

The findings presented in Table 2 show that the MPO, HNL and ECP values correlated with the mucosal structural changes and the increase of chronic inflammatory infiltrate. The presence of neutrophils in lamina propria and in epithelium correlated with the neutrophil markers MPO and HNL and the presence of eosinophils in lamina propria correlated with the eosinophil marker ECP. The histological findings in patients with IBS were characterized by no increase or mild but unequivocal increase of eosinophils in lamina propria. The correlations observed between the number of mucosal neutrophils/eosinophils and neutrophil/eosinophil markers in patients with UC remained when calculations were performed in all patients.

Table 2: Correlation coefficients between mucosal inflammatory mediators and histological grades of disease activity in patients with ulcerative colitis.

	MPO	HNL	ECP
Structural (architectural change)	0.75**	0.75**	0.80**
Chronic inflammatory infiltrate	0.77**	0.79**	0.76**
Lamina propria eosinophils	0.46	0.48	0.64*
Lamina propria neutrophils	0.75**	0.77**	0.61*
Neutrophils in epithelium	0.55*	0.59*	0.38
Crypt destruction	0.41	0.45	0.22
Erosion or ulceration	0.49	0.57*	0.40

*p<0.05 and **p<0.01 according to Spearman Rank Correlation

Serum measurements

The serum concentration of HNL in patients with active UC was significantly higher than those in patients with IBS and coeliac disease and healthy controls (p<0.05) However, the differences in serum values of MPO, ECP and HNL were relatively marginal compared to the observed differences in mucosal measurements.

Kinetic of rectal mucosal granulocyte activation in patients with coeliac disease before and after rectal gluten challenges. (Study II)

Early gluten reaction studied by the use of rectal perfusion technique:

During the basal rectal perfusion, patients and controls had similar concentrations of MPO, ECP and histamine in the perfusion fluid (Figure 7). Three hours after gluten challenge a progressive, significant increase in the MPO concentration was seen during the next 3 hours (Figure 7). The maximum MPO concentration increased on average from ≤ 3 to 123 ± 142 (SD) $\mu\text{g/l}$ at 6 hours after challenge ($p < 0.001$). The ECP concentration started to increase at a later time point (4 hours after gluten challenge). The ECP increase was less

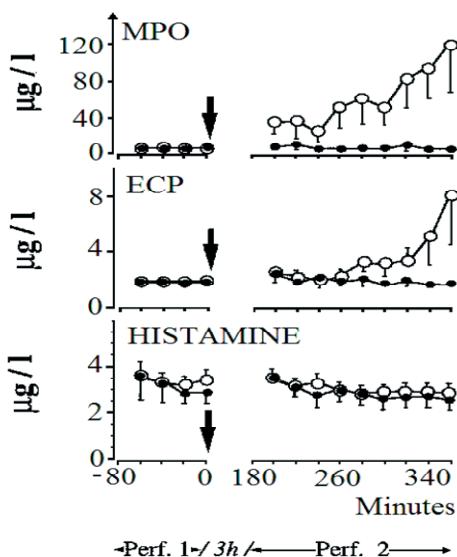


Figure 7. Myeloperoxidase (MPO), eosinophil cationic protein (ECP) and histamine concentrations in rectal perfusion fluid (mean \pm SE) before and after a rectal gluten challenge in 5 healthy controls (-●-) and 8 patients with coeliac disease (-○-). The arrow indicates the time for gluten challenge. Perf.1 is the pre-challenge perfusion and Perf.2 the post-challenge perfusion.

pronounced and reached a maximum at the end of the perfusion; an average increase from ≤ 3 to 8.0 ± 10.5 (SD) $\mu\text{g/l}$ ($p < 0.05$). In only one patient there was a significant, 50-fold increase in histamine concentration post-challenge (Figure 8). In the controls, no significant differences between the pre- and post-challenge concentrations of the measured inflammatory mediators were observed.

Late gluten reaction studied by the use of the mucosal patch technique:

Figure 9 shows the rectal mucosal concentrations of MPO and ECP in patients with coeliac disease (n=10) before and at 5, 15, 24 and 48 hours after rectal gluten challenge. The prechallenge values of MPO found in the healthy controls were 9.5 ± 6.4 (SD) $\mu\text{g/l}$, i.e. significantly lower ($p < 0.05$) than those in the patient group. Significant increases in the variables measured were seen at every observation time of MPO. The pattern was similar for ECP and MPO, with a peak level 5 hours after challenge followed by a decline at 15 hours and another phase of increasing levels thereafter. The MPO concentration then seemingly peaked 24 hours after challenge and started to fall after 48 hours. The ECP concentrations remained at the same increased levels 24 and 48 hours after challenge. The healthy controls (n=15) showed no significant change in the measured variables when tested before and 15 hours after gluten challenge (data not shown). The rectal mucosal histamine levels 5 and 15 hours after challenge were similar to the prechallenge values both in the patients and controls (data not shown).

The serum concentrations of MPO and ECP were similar in patients and controls both before and after challenge.

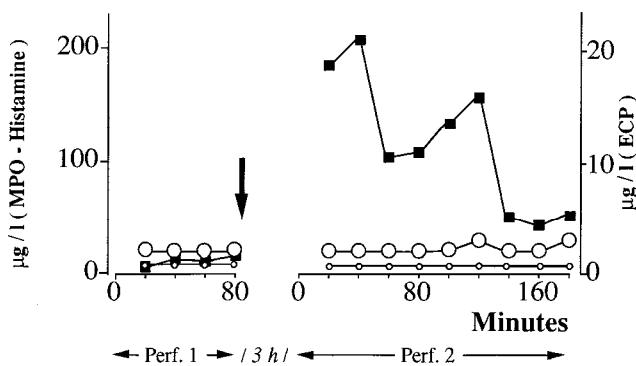


Figure 8. The rectal perfusion fluid concentration of myeloperoxidase (-○-), histamine (-■-) and eosinophil cationic protein (-○-) before and after a rectal gluten challenge in patient nr 3 with recently diagnosed coeliac disease. The arrow indicates the time for gluten challenge. Perf.1 is the pre-challenge perfusion and Perf.2 the post-challenge perfusion

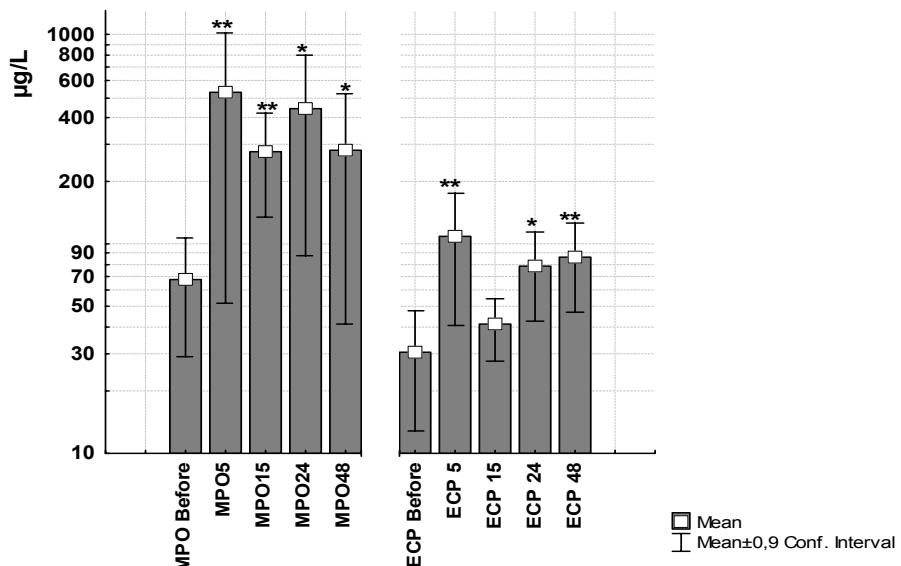


Figure 9: The rectal mucosal concentrations (Means \pm SEM) of MPO and ECP before and 5, 15, 24 and 48 hours after gluten challenge in patients with coeliac disease (n=10). *p<0.05, **p<0.01 compared to the prechallenge values (Sign test)

Rectal luminal NO production after rectal gluten challenge in patients with coeliac disease and the relation to granulocyte activation (study III)

The prechallenge rectal luminal NO value in the patients with CD was 19 ± 4 ppb (6-41) and in the controls it was 24 ± 6 ppb (6-90). No increased NO production was detected 5 hours after challenge. The maximum NO concentration in patients 9464 ± 2393 ppb, (250-24982) was noted after 15 hours. The NO values then gradually declined but had not returned to normal in all patients at 48 hours. The mean NO production in the controls 15 hours after rectal gluten challenge was 22 ± 7 ppb (4 – 126).

Relation of NO production to signs of granulocyte and mast cell/basophil activation

There was a significant correlation ($r=0.64$, $p<0.05$) between the increases in MPO (Δ MPO) and the increase in NO (Δ NO) 15 hours after challenge. No correlation was found between the Δ ECP and Δ NO 5 and 15 hours after challenge, r values 0.02 and 0.04, respectively (Figure 10).

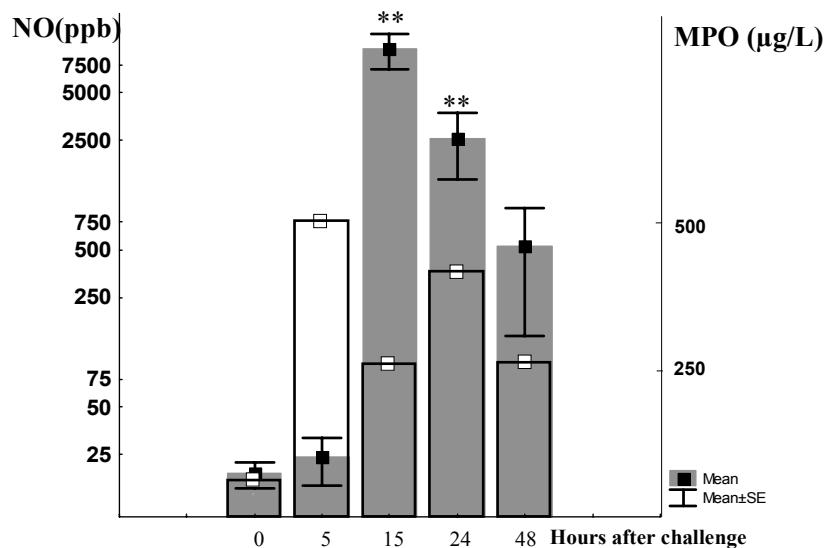


Figure 10. Rectal luminal NO before and 5, 15, 24 and 48 hours after rectal gluten challenge in 10 patients with coeliac disease. Friedman ANOVA was significant ($p<0.001$), ** $p<0.01$ compared to prechallenge value (Sign test). The open squares illustrate the rectal mucosal MPO concentrations at each time and the relationship to NO production

Corn and Cow's milk protein sensitivity in coeliac disease (Study III and IV)

Corn sensitivity

In the patients with coeliac disease the NO production 15 hours after corn challenge was significantly increased by a mean of 368 ± 147 ppb (13-1348) compared to the pre-challenge value ($p<0.05$). However, as seen in Figure 14, only 6/10 patients showed a post challenge increase in NO. In the controls no increase of NO was seen after corn challenge (Figure 11.)

Corn challenge also induced an increase of MPO in some of the patients but the increase was not significant in the group ($p>0.05$). No increase was seen in ECP after corn challenge. The control subjects showed no increase of MPO or ECP after challenge with corn.

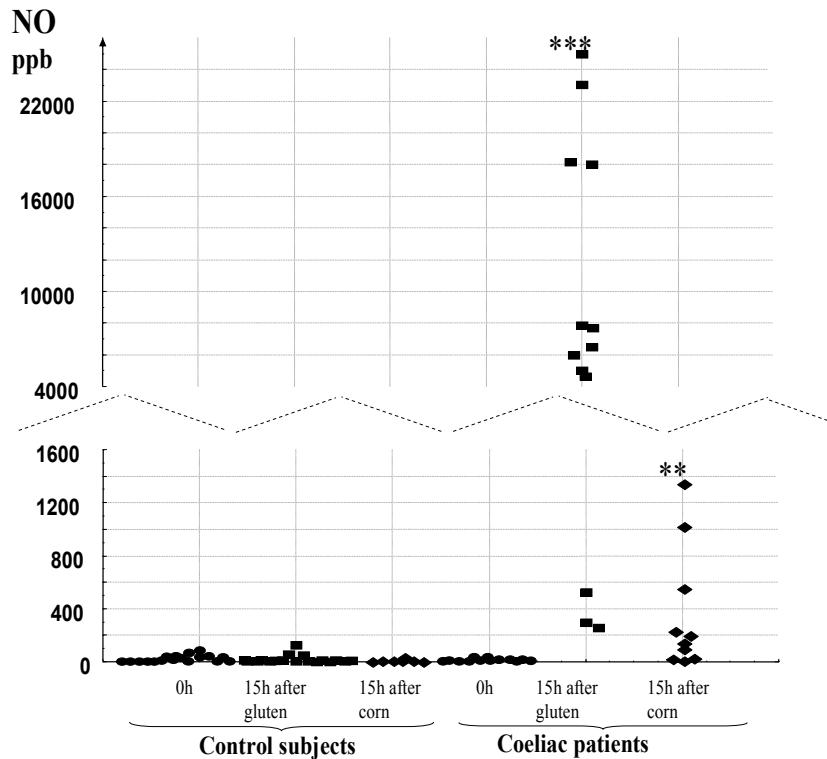


Figure 11. Rectal concentrations of NO in 18 controls and 13 coeliac patients before challenge (0h), 15 hours after rectal wheat gluten challenge (15h after gluten) and 15 hours after rectal corn gluten challenge (15h after corn). Note that the Y scale is broken and differs between section below and above the break in order to better illustrate the lower values. Significant increase comparing the patient and control groups with Mann-Whitney U Test: *** $p<0.001$, ** $p<0.01$

Milk sensitivity

In 18/20 patients gluten challenge induced neutrophil activation defined as increased MPO release and increased NO synthesis. A significant increase for one individual was defined by value above the mean $\Delta\text{MPO}/\Delta\text{NO} + 2 \text{ SD}$ level in the controls. Ten of these 20 patients showed a similar strong inflammatory reaction to cow's milk challenge. ECP was increased to a similar extent in the patient group and the controls. Figure 15 illustrates the individual increases in NO and MPO after cow's milk challenge. In the group of coeliac patients the rectal NO and MPO values correlated both after cow's milk ($r=0.73$, $p<0.001$) and gluten challenges ($r=0.54$, $p<0.05$). No correlation was found between ECP and the other variables after challenge.

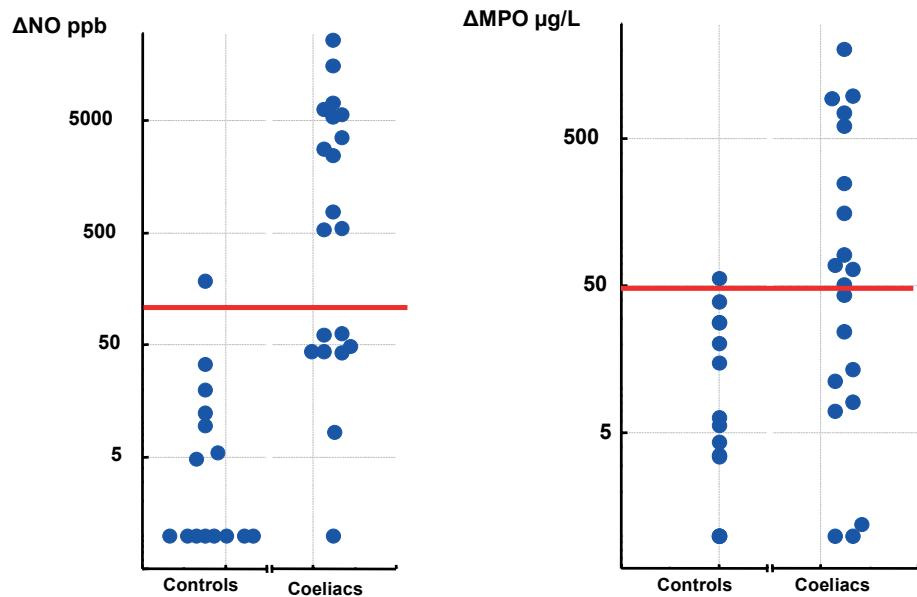


Figure 12 Increase in rectal luminal nitric oxide (ΔNO) and rectal mucosal concentration of MPO (ΔMPO) in patients with coeliac disease (n=20) 15 hours after rectal milk challenge. The level of two standard deviations (SD) above mean of the control subjects (n=15) is marked by a line.

IgA and IgG antibodies to cow's milk proteins. The mean serum levels of IgA and IgG antibodies to gliadin, casein, and α -lactalbumin were not higher in the patients with CD than in the controls

Challenge with casein and α -lactalbumin. Six patients reactive to CM were challenged with casein and α -lactalbumin (Table 3). All showed an increase in MPO and/or NO in responses to casein and one patient (no. 3) had a significant increase in ECP. The α -lactalbumin caused an MPO response in one patient. Most of the patients reported subjective clinical improvement of symptoms (diarrhea, abdominal distension / pain or tiredness) after being informed about the results and excluding CM protein from their diet (Table 3).

Table 3: Reactivity to casein and α -lactalbumin at rectal challenge in six patients sensitive to cow's milk as defined by significantly elevated Δ NO and/or Δ MPO values after challenge. The clinical effect of exclusion of cow's milk protein from the diet on the most common symptoms mentioned (diarrhea, abdominal distension/pain and tiredness) is shown given on a four-graded subjective scale (0 representing no improvement and 3 resolution of symptoms).

Case	Casein	α -Lactalbumin	Clinical effect
	NO/MPO	NO/MPO	
1	+/-	-/-	1
2	(+)/++	-/-	0
3	+/+++	-/++	2
4	+++/+++	No value	1
5	+++/++	-/-	2
6	+++/++	-/-	3

(+)= a value between the upper and two times the upper 99% confidence interval (CI) of the controls; (++)= a value between two and five times the 99% CI; (+++)= a value above five times the 99% CI.

General discussion

The Mucosal patch technique

This study presents the new mucosal patch technique for measuring markers of events in the colorectal mucosa and shows that it is simple to use, safe and very reliable and that the patient compliance is good. Compared to other methods,^{43,44,54,137,138} the present system has several advantages. In our experience it is technically simpler to perform and more comfortable for the patients than rectal dialysis or rectal perfusion techniques.^{43,44} The size of the molecule to be evaluated is not critical, as it is in rectal dialysis. It does not require an endoscopic procedure, as in rectal perfusion and in the filter technique described by Hendel and co-workers, but it can easily be combined with endoscopy when required.

Studies of the time needed for the cellulose material to absorb inflammatory substances from the mucosa indicated that after less than 20 minutes, equilibrium between the mucosa and cellulose was reached. For practical clinical purposes it seems quite acceptable to reduce the time of contact between the cellulose material and the mucosa to about 10 minutes.

Preparation of the gut is essential in situations where the release of inflammatory substances to the gut lumen is measured, since faecal contamination may influence the results.¹³⁹⁻¹⁴¹ It has also been reported that hyperosmolar solution might affect the mucosa if the osmolarity is over 1500 mosm/L.¹⁴² The enema we used has an osmolarity of 1686 mosm/L. However, preparation with an isotonic fluid and preparation with a hyperosmolar enema gave identical results with the present system and neither method induced any apparent inflammatory reaction in the rectal mucosa.

Patchiness of mucosal inflammation in UC has previously been emphasized.^{143,144} In an attempt to compensate for possible regional differences in the inflammatory status of the rectal mucosa, we performed three measurements at different rectal sites. The average intraindividual variation of three rectal measurements was 38 %, thus illustrating the expected variability in a mucosal inflammatory process. The difference in the mean of such measurements performed at different times was not significant. Thus, the reproducibility was quite acceptable with this process, which means that longitudinal studies of the condition of the mucosa can be made in individual patients.

Patients with active ulcerative colitis showed on average a more than 300-fold increase in the inflammatory activity of the rectal mucosa as judged by the recovered amounts of MPO. The involvement of eosinophils in this disease was also clear from the several-fold increase in the recovery of ECP, and our findings are in accordance with our previous report using a perfusion technique.¹⁵ Patients with inactive ulcerative colitis showed normalisation of the eosinophil activity of the mucosa, but the neutrophil activity remained increased compared to that in healthy controls but of the same magnitude as observed in IBS and coeliac disease. The potential of this sensitive method for assessment of inflammatory activity was also illustrated by the significantly increased recovery of neutrophil granule constituents in our patients with collagen colitis. In a minority of these patients the mucosal recovery of ECP was highly increased in accordance with recently published data from a study using rectal perfusion.¹⁴⁵

By virtue of the extreme absorption capacity of the cellulose material used for the test procedure the mucosal patch technique is very sensitive and permits detection of neutrophil and eosinophil granulocytes in apparently healthy rectal mucosa. In this study subclinical inflammation in the rectal mucosa was detected in patients with IBS. The inflammatory basis of IBS has been discussed, and non-specific mild inflammation in the colonic mucosa is often seen histologically in IBS patients.¹⁴⁶⁻¹⁴⁸ It has been proposed that such mucosal inflammatory findings might be explained by an undiagnosed coeliac disease or a postinfective IBS.^{148,149} However, such possible explanations are not relevant for our findings in IBS since only two of our IBS patients had a history of an IBS secondary to a gastrointestinal infection and all our IBS patients had normal duodenal biopsies and were negative in serological markers of coeliac disease.

Chadwick et al¹⁴⁸ promoted the hypothesis that in at least a subset of patients with IBS a low-grade inflammation with increased numbers of neutrophils and mastcells might play a pathophysiological role. Others¹⁵⁰ have reported, in this disease, increased numbers of mast cells in proximal colonic mucosa but no increase of these cells or neutrophils in the rectum. Since overt colonic inflammation precludes a diagnosis of IBS, the need of more sensitive markers of low-grade inflammation has been stressed.¹⁵⁰ Counting of single inflammatory cell subtype per high power field has the potential to be more sensitive compared to our used histological method validated for UC to assess the degree of mucosal inflammation. Conventional histological examination of biopsies identifies the number of inflammatory cells but give no information about the degree of granulocyte activation. Increased release of granular constituents is one sign of granulocyte activation. Our finding of increased mucosal amounts of neutrophil granular proteins (MPO and HNL) in spite of no apparent increase of neutrophils might therefore reflect a low grade inflammation. This interpretation is supported by the finding that IBS patients have significant increased amounts of faecal calprotectin, a neutro-

phil derived protein.¹⁵¹ The correlation between measured inflammatory mediators and the endoscopic and histological findings in UC indicate that our technique mainly reflects a local inflammatory process.

Kinetics of inflammatory reaction seen after rectal gluten challenge

This study confirms previous histological observations on mucosal biopsy specimens showing that a response to rectal gluten challenge occurs both in untreated and diet-treated coeliac disease patients.^{4,5,48-50} The mechanisms by which wheat gliadin and prolamines from other cereals produce lesions in the small intestine of patients with coeliac disease are not completely understood. Most investigators favour the idea of coeliac disease as a prototype of T-cell mediated disease.¹⁵² Thus, gliadin should act as an antigen recognized by the adaptive immune system. However, the situation may be more complex since the immune reactions can be further driven by gliadin induced activation of the innate immune system⁸⁰ Studies on the rectal mucosal reaction have mainly been focused on epithelial lymphocyte responses. Besides a marked increase in epithelial lymphocytes with a maximum at 6-8 hours post-challenge infiltration of neutrophils into the mucosa is reported.^{4,5,52} An apparent decrease in mast cells has been attributed to granule discharge and thereby loss of recognizable specific cytoplasmic granules.⁴ Involvement of eosinophils has previously been reported from studies with regional jejunal perfusion of patients with active coeliac disease.¹⁵³ One of the aims of our studies was to further elucidate the gluten-induced mucosal reaction by defining the dynamics of the leucocyte and mast cell/basophil activation. We found signs of pronounced neutrophil activation and of more modest eosinophil activation, and only in exceptional cases signs of mast cell/basophil degranulation.

In patients with coeliac disease the studies with continuous rectal perfusion revealed an increase in the MPO concentration in the perfusion fluid as early as three hours after gluten challenge. During the following 160 minutes there was a progressive rise in MPO. At the end of the perfusion period the MPO concentration had increased on average 40-fold. The ECP concentration started to increase later, and almost 6 hours after gluten challenge the increase was significant. These findings indicate that gluten challenge in coeliac disease induces a major neutrophil and a more modest eosinophil accumulation in the rectal mucosa and release of their granule constituents. As with the perfusion technique the mucosal patch technique confirms a large increase in MPO 5 hours after gluten challenge. Ten hours later the MPO concentration had declined, but a further increase was observed at 24 hours after challenge, suggesting biphasic neutrophil activation. A similar

biphasic pattern was seen for eosinophil degranulation. These results support results of previous studies. Loft et al observed biphasic swelling of the lamina propria following rectal gluten challenge in patients with coeliac disease.⁴ They reported an initial swelling period with a maximum 8 hours after challenge, followed by deswelling after 24 hours and another swelling peak at 60 hours post challenge. They also found a similar biphasic influx of neutrophils and epithelial lymphocytes. The biphasic response might reflect that the gluten-induced reaction involves the adaptive as well as the innate immune systems.⁸⁰ In another study the time course of adhesion molecule expression in the rectal mucosa of gluten-sensitive subjects was followed during a 24-hour period after gluten challenge.⁵² A significant increase in adhesion molecules related to neutrophils was seen in the lamina propria 4 hours after challenge.

In the above-mentioned study by Loft et al a rapid decrease in the number of lamina propria mast cells appeared to occur within 1 hour after gluten challenge.⁴ This effect was assumed to be due to granule discharge and hence loss of recognizable specific cytoplasmic granules on which microscopic quantisation was based. However, in our study only one of our patients showed signs of degranulation of mast cells/basophils as indicated by an increased luminal release of histamine in response to gluten challenge. Thus, according to our results mast cell activation may occur but only in a subgroup of coeliac disease patients. Our histamine responder had no IgE-anti-gliadin antibodies. She had high titres of IgA and extremely high titres of IgG wheat gluten antibodies. The possibility remains that IgG antibodies and IgG-gliadin complexes may have contributed to mast cell activation.¹⁵⁴⁻¹⁵⁷

Before challenge, most of our patients had higher mucosal concentrations of MPO but not of ECP compared to the controls. This increase in the basal concentration could reflect either a normal-sized but activated neutrophil population or an expanded population of non-activated cells, or a combination of these two possibilities. The idea of subclinical rectal inflammatory activity is not improbable, since apparent proctitis has previously been reported in patients with coeliac disease.^{158,159} Occurrence of subclinical rectal mucosal inflammation in coeliac disease even after introduction of a gluten-free diet might be explained by an enhanced non-specific inflammatory propensity or to the small amount of gluten received in a normal gluten-free diet, or possibly by poor compliance. Further, the possibility remains that coeliac disease patients may react to other proteins than wheat gluten.¹²¹

Previous studies with multiple jejunal mucosal biopsies after reintroduction of gluten to coeliac patients have demonstrated a considerable interindividual variation with respect to the severity and time to clinical and histopathological relapse.¹²⁰ Our findings clearly demonstrate that the individual degree of inflammatory reaction, as indicated by rectal mucosal release of MPO, varies within a wide range. It is reasonable to assume that the intensity of the gluten-induced rectal mucosal inflammation should be reflected in the

patient's symptoms. The heterogeneity of symptoms in patients with untreated coeliac disease is also well known, as also is the variation in individual sensitivity to minor amounts of gluten.

Kinetics of NO production after rectal gluten challenge

Our main finding was that signs of granulocyte activation clearly preceded the increased synthesis of NO after gluten challenge. Increased NO synthesis was not apparent 5 hours after gluten instillation, a time point with maximum release of MPO, a granule constituent of neutrophils, and of ECP, a granule constituent of eosinophils. The luminal NO values peaked 15 hours after gluten challenge and then gradually declined, but were still increased after 48 hours.

The role of NOS II in the inflammatory response to gluten instillation in the rectal mucosa has previously been elucidated by measurements on rectal mucosal biopsy samples obtained 4, 8, 24 and 48 hours after rectal gluten challenge.¹⁶⁰ A significant, approximately 50% increase in NOS II was seen 8 hours after gluten challenge, but no change in cNOS. Increased NO production in the gut lumen has been observed not only in ulcerative colitis,¹⁶¹ but also 24 hours after gluten challenge in patients with CD.¹⁶² Our finding of high production of NO starting 5 to 10 hours after gluten challenge is in accordance with the above mentioned biopsy results concerning NOS II expression,¹⁶⁰ since this expression has been found to precede the production of NO by several hours; the delay in NO production reflecting the time taken for mRNA and protein synthesis.^{163,164} The NOS II expression in rectal biopsy specimens gradually decreased and was lost 24-48 hours after challenge. Our NO measurements showed a return to prechallenge NO values in 30% of our patients, but elevated NO values still remained in a few patients even 48 hours after challenge. In bronchial asthma an increase in luminal airway NO is also seen and it has been suggested that this may be useful for a non-invasive determination of airway inflammation.¹⁶⁵ After allergen challenge in asthmatic patients, no increase of NO was observed in those who responded only with an acute reaction, while those, who also had a late allergic response, had a peak increase of NO at 10 hours and still showed elevated NO levels 21 hours after challenge. The similarity to our NO reaction strengthens the idea that the gut and airway luminal NO production might be a non-specific inflammatory response.

The cellular source of induced luminal NO synthesis has not been fully identified in asthmatic patients. There is evidence of increased expression of NOS II in asthmatic airways, especially in macrophages and epithelial cells, but activated neutrophils, eosinophils and mast cells have also been proposed as major contributors to enhanced NO synthesis.¹⁶⁶⁻¹⁶⁸ The early inflammatory reaction induced by gluten in patients with CD is characterized by ex-

pression of E-selectin, a specific adhesion molecule that is the main mediator involved in neutrophil recruitment in the first 4 hours after gluten exposure.⁵² This is in concordance with our observations that a strong neutrophil activation, as assessed by the luminal release of MPO, and less pronounced eosinophil activation, as measured by the release of ECP, was already present five hours after gluten instillation challenge and clearly preceded the NO response.

The observed interindividual variability in NO and MPO responses might partly be due to non-homogenous inflammation. Another, perhaps more likely explanation to this variability is the individual sensitivity.¹²⁰ The finding of a significant correlation between the neutrophil and NO responses after challenge demonstrates that NO synthesis is linked to the intensity of the induced inflammatory reaction, but only suggests that neutrophils are the cellular NO source.

Nitric oxide in exhaled air (eNO) has been proposed as a marker of bronchial inflammation. Some studies reported significant association in patients with bronchial asthma between eNO and airway eosinophilia as reflected by sputum eosinophilia^{169,170} or density of eosinophilic granule constituents in airway mucosa. Because eosinophils can express NOS II¹⁷¹ these cells have been attributed a role in the synthesis of NO after allergen challenge. However, others have been unable to confirm a relationship between eNO and airway eosinophilia in asthma.^{172,173} In the present study signs of eosinophil activation as reflected by ECP were present after gluten challenge in patients with coeliac disease with a similar time course of activation as neutrophils but the eosinophil response was relatively weaker than the neutrophil response defined by MPO. The lack of relationship between the degree of eosinophil activation and NO production may suggest that NO reflects other aspects of gut mucosal inflammation, including neutrophil and possibly also T-cell and monocyte activation.

The site of NOS II expression in the epithelium of gluten-damaged rectal mucosa is a matter of controversy, but immunohistochemical studies of such mucosa showed that NOS II was mostly localized in the lamina propria just beneath the surface epithelium and around the crypts.¹⁶⁰ The suggested protective effect of NO in inflammation has been partly attributed to a role in reducing granulocyte infiltration and consuming oxygen species.³⁷ Thus, the observed relationship between signs of neutrophil activation and later NO synthesis may well reflect a counteracting system controlling the potentially tissue-damaging principles delivered by activated neutrophils. However, the reports suggesting a protective role of NO in mucosal inflammatory damage are as numerous as those supporting a toxic role. It has been proposed, for example, that NO may promote inflammation by enhancing vascular permeability and by promoting chemotaxis of granulocytes and the production of proinflammatory cytokines.^{174,175} Others have suggested that epithelial cells

are the cellular source of luminal nitric oxide seen in bronchial asthma and inflammatory bowel diseases.^{176,177} Our kinetic study is compatible with this hypothesis, since activated granulocytes may induce enterocytes to increase NO synthesis. The results of our study give no answers concerning the possible toxic or protective role of nitric oxide in gluten-induced damage. The possibility also remains that the association between granulocyte activation and nitric oxide production merely reflects the metabolic expression of neutrophils/eosinophils and other inflammatory cells activated by gluten challenge.

The lack of luminal histamine release after gluten challenge indicates that mast cells/basophils are not involved in the induced NO synthesis. Niviloni et al observed in biopsy samples that the number of NOS II-positive cells after gluten exposure increased in parallel with the CD3 cell infiltration observed around the subepithelial areas.¹⁶⁰ T-cells have been attributed a central role in the pathogenesis of gluten-induced mucosal damage, but the NO-producing capacity of T-cells in inflammation remains unsolved.

Food hypersensitivity in coeliac disease

Corn gluten

The observation that corn gluten challenge induced an abnormal NO reaction in some of our patients with coeliac disease is intriguing, since maize is considered safe and is recommended as the substitute cereal in a gluten-free diet. However, a high incidence of serum antibodies against maize¹⁷⁸ has been reported in coeliac disease, and the failure to normalise the mucosa in a fraction of adult patients with coeliac disease and on a strict wheat gluten-free diet remains to be explained.¹²¹ The manufacturer claimed that their corn product was free from wheat or other cereals. We tested the product at the Swedish National Food Administration (Livsmedelsverket) and it was found to be contaminated with an amount of 82 microgram per gram (ppm) which is less than the usual allowed amount in gluten free diet (<200ppm) according to Codex Alimentarius Standard for gluten-free foods, and far less than what has been found to be a safe amount of gluten contamination, when correlated to histology, in oral challenge studies.¹¹⁸ It can not be excluded that the small amounts of gluten present in the used corn preparation have induced an inflammatory reaction since the mucosal patch technique is very sensitive.

Milk protein

Ten of our 20 patients with coeliac disease showed abnormal increases in both MPO and NO as a reaction to cow's milk protein challenge, but no increase in ECP, indicating absence of eosinophil activation.

Our 20 patients were already on a gluten-free diet at the time of the present study and 9 of them had persistent minor mucosal abnormalities. Failure to normalize the mucosa has been attributed to the fact that complete elimination of gluten is very difficult to achieve and maintain. The trace amounts of gluten allowed according to the Codex Alimentarius Standard for gluten-free foods have also been suggested as a cause of this lack of total recovery.¹²¹ However, others have claimed that persistent mucosal abnormalities in CD are not related to the ingestion of trace amounts of gluten.¹²² but have proposed that other food components, especially cow's milk proteins, may induce food reactivity in some coeliac patients.^{121,123-125}

The major food antigens in coeliac disease are gliadin and similar prolamines from rye and barley. In active disease increased serum antibodies not only against gliadin but also against cow's milk proteins are seen.¹⁷⁹ However, direct evidence for cow's milk protein allergy in CD is lacking. Most exposed healthy individuals have low levels of antibodies against various food antigens.^{179,180} The likely explanation of this physiological phenomenon is that a small fraction of food proteins passes undegraded across the gut barrier⁷⁶ and thereby presents to the immune system with subsequent production of antibodies. Certain diseases are characterized by enhanced antibody production against dietary antigens. The elevated levels of IgG and IgA anti-cow's milk protein antibodies observed, for example, in coeliac disease¹⁸¹ and inflammatory bowel disease¹⁸² could be related to the damaged intestinal mucosa, causing increased penetration by undegraded proteins.¹⁸³ Our patients with coeliac disease had normal serum levels of IgA, IgG and IgE against casein and α -lactalbumin, which might be explained by the fact that they were on a gluten-free diet and therefore had improved the mucosal integrity.

Tissue transglutaminase 2 has been identified as the autoantigen in coeliac disease¹⁰⁰ and IgA anti-tTG autoantibodies are very sensitive markers for this diagnosis.¹⁸⁴ Both the expression of coeliac disease and the presence of serum antibodies to tTG are strictly dependent on dietary exposure to gluten.^{100,184} Extra cellular tTG can cross-link glutamine-rich proteins, particularly gluten from wheat, in which glutamine constitutes about 40% of the amino acids. Casein is also glutamine-rich and is known to bind to tTG.⁶⁶ The observation that the production of an autoantibody is dependent on the intake of a dietary protein such as gluten may seem confusing, but has been explained by the existence of antibodies directed against cross-links between gliadin and tTG.^{66,185} Antibodies to gliadin seem to be an epiphenomenon in coeliac disease and the pathophysiological role of auto antibodies to tTG

remains unclear. Our results in celiac patients on a gluten free diet demonstrate that gluten and casein challenges induce a gut mucosal inflammation by pathways until now not identified but at least antibody independent. Recently it has been demonstrated that certain gluten peptides elicit not only an adaptive but also an innate immune response.^{80,186} The innate immune system provides an early, so-called pattern-recognition response to various tissue-damaging agents, e.g. viral proteins and bacterial DNA. In individuals with the genetic prerequisites, an innate response to normally harmless dietary proteins might precede and enhance adaptive immunity to such proteins. Activation of the adaptive immune system is one prerequisite for the occurrence of coeliac disease and is reflected by the development of gliadin antibodies and auto antibodies. Our finding that in 50 % of our celiac patients casein challenge induced an inflammatory reaction of the same magnitude as did gluten challenge suggests an innate response to casein also. The lack of increased serum antibodies to casein in our casein-sensitive celiac patients suggests that casein is less prone than gliadin to drive adaptive immunity. Nevertheless there are certain similarities between gliadin and casein: Both proteins are chemotactic to human leukocytes^{187,188} and are stable to digestion because of their content of praline.⁷⁵ They also show certain amino acid sequence homologies.¹⁸⁹

The classic presentation of coeliac disease with diarrhoea and malabsorption is relatively rare, whereas atypical, oligosymptomatic or even asymptomatic coeliac disease but with histological signs of inflammatory enteropathy is frequently seen in the western countries, with an estimated prevalence of 1:100-200. Coeliac disease is strongly associated with certain HLA class II alleles, 90% of the patients being HLA DQ2⁶⁶ Additional genes may relate to the recently observed innate immune reactivity to certain gliadin peptides¹⁹⁰ In the light of the hypothesis that the casein sensitivity found in coeliac disease also reflects an innate response, we have to consider the possibility that sensitiveness to gluten and casein share common genes related to innate immunity.

Several studies have demonstrated, mainly in adult patients, a close association between coeliac disease and certain other autoimmune disorders, especially insulin-dependent diabetes, autoimmune thyroiditis and Sjögren's syndrome.¹⁹¹ Other researchers have suspected that cow's milk protein, also, and especially casein, may have a harmful effect as an environmental trigger of various autoimmune diseases.¹⁹²⁻¹⁹⁴ Our preliminary observations in the present study suggest that elimination of cow's milk protein in coeliac disease patients with casein sensitivity may improve their gastrointestinal symptoms. Objective means to diagnose such sensitivity in patients with other autoimmune diseases are required to be able to elucidate the question whether or not gluten and cow's milk protein may also aggravate their symptoms.

Conclusions

- The mucosal patch technique was well tolerated by the patients and easily applied by the investigator. Pronounced neutrophil and eosinophil involvement in ulcerative colitis was demonstrated. With the high sensitivity of the technique, low-degree mucosal neutrophil activation could also be quantified in patients with collagen colitis and ulcerative colitis in clinical remission. The finding of increased neutrophil involvement in patients with IBS contributes to the pathophysiological ideas of this disease.
- There is a pronounced neutrophil activation in coeliac patients after rectal gluten challenge. This activation is apparent 4 hours after challenge and remains for at least 48 hours. A more modest eosinophil activation defined by ECP levels starts 1-2 hours later and remains also at least for 48 hours. The biphasic pattern of MPO and ECP after challenge suggests a biphasic inflammatory reaction.
- Mucosal activation of neutrophils and eosinophils precedes a pronounced enhancement of mucosal NO production after rectal wheat gluten challenge in patients with coeliac disease.
- The findings indicate that not only gluten sensitivity but also cow's milk protein sensitivity is common in coeliac disease. The data support the hypothesis that cow's milk sensitivity may contribute to persistent symptoms in coeliac patients on gluten-free diet.

Clinical implication and future

The mucosal patch technique is a new principle for assessing biological events in the colonic mucosa. This technique is rapid, simple, safe and highly sensitive and produces reliable and reproducible data. The results obtained in our studies show that it might be suitable not only for research studies of gut pathophysiology but also for practical clinical use in various diseases involving inflammatory activity in gut mucosa. It has a potential to be a valuable tool in the evaluation of the time course and treatment of these diseases. Studies with measurements of various other mediators involved in these diseases are also possible.

Our studies show that rectal provocation with gluten combined with measurement with the mucosal patch technique is a potential clinical practical tool to elucidate the possible role of gluten in patients who refuse to or are not able to have biopsies taken during endoscopy. By use of the technique we can also evaluate the roles of other food antigens, such as milk in patients with coeliac disease. These findings are also promising regarding research and diagnostics of food hypersensitivity in other patient groups.

The high sensitivity of the technique with findings of increased neutrophil involvement in patients with IBS contributes to the pathophysiological ideas of this disease and is worth a special comment. Between 3% and 22 % of persons in the community have IBS with lower quality of life and the economic impact is enormous.¹⁹⁵ The pathophysiology and etiology of IBS is not known and treatment is symptomatic. Further studies of various mediators in the gut mucosa in these patients are necessary to try to elucidate if there are subgroups with treatable inflammatory activity.

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