CYTOTOXIC LYMPHOCYTES IN CHILDREN'S COW'S MILK SENSITIVE ENTEROPATHY OF DELAYED TYPE

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2005
Oulu, Finland

Abstract

Food hypersensitivities are becoming increasingly common worldwide. Previous studies indicate that cell mediated immunity has a role in delayed paediatric gastrointestinal food hypersensitivities, but the exact pathogenetic mechanisms are unknown. Cytotoxic activation of T-lymphocytes is known to play an important role in the pathogenesis of celiac disease (CD). The pathogenetic mechanisms of cow’s milk protein sensitive enteropathy (CMSE) are largely unknown. CMSE is a non-IgE related type of food hypersensitivity with variable gastrointestinal symptoms but no visible mucosal abnormalities on light microscopy. The diagnosis is based on an open or blinded elimination/challenge test, as the endoscopic, histological and laboratory findings are generally non-specific.

This thesis aims to characterize the role of lymphocyte cytotoxicity in the pathogenesis and diagnosis of CMSE in preschool and school aged children, including comparison with CD where the pathogenetic significance of cytotoxicity is well established. The study cohort consisted of 151 children, including 57 with untreated CMSE, 18 with treated CMSE, 24 with CD, and 52 controls. Using immunohistochemistry, the mucosal expressions of cytotoxic T cell-restricted intracellular antigen type 1 (TIA-1), perforin, granzyme A and B were analysed in the duodenal bulb and descending duodenum. Intraepithelial T-lymphocytes were labelled with CD3, alpha/beta and gamma/delta T cell receptor antigens. To determine the rates of overall and epithelial apoptosis as well as proliferation, the immunohistochemical TUNEL technique, M30 and Ki-67 antibodies were used. Serum levels of granzymes, CD30 and soluble Fas were studied using ELISA method.

The number of intraepithelial lymphocytes with TIA-1, perforin and granzyme A containing granules was increased in CMSE. This increase was related to antigen challenge and not a constitutional abnormality. The cytotoxic reaction in CMSE differed from that in CD by being of lesser magnitude, concerning predominantly the descending duodenum and not showing signs of cytotoxicity related epithelial destruction. The serum levels of GrA, GrB and CD30 were increased in both CMSE and CD, correlating with the number of duodenal CD3⁺, alpha/beta and gamma/delta⁺ intraepithelial lymphocytes.

The results strongly support the role of cell-mediated immunity in the pathogenesis of CMSE. Mucosal cytotoxic activation seems to be manifested by the release of cytotoxicity related proteins in serum. This provides a new approach to the monitoring of intestinal immune activation which could help in diagnosis and in objectively monitored treatment response.

Keywords: apoptosis, biopsy, celiac disease, cow's milk protein sensitive enteropathy, duodenum, granzyme, lymphocyte, mucosa, perforin, serum, TIA-1
To my sweet daughter Maisa
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Oulu, May 2005 Merja Augustin
# Abbreviations

<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AICD</td>
<td>activation-induced cell death</td>
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<tr>
<td>APAF-1</td>
<td>apoptotic protease-activating factor-1</td>
</tr>
<tr>
<td>APCs</td>
<td>antigen-presenting cells</td>
</tr>
<tr>
<td>APT</td>
<td>atopy patch test</td>
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<tr>
<td>CD</td>
<td>celiac disease</td>
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<tr>
<td>CLs</td>
<td>cytotoxic lymphocytes</td>
</tr>
<tr>
<td>CMA</td>
<td>cow’s milk allergy</td>
</tr>
<tr>
<td>CMPI</td>
<td>cow’s milk protein intolerance</td>
</tr>
<tr>
<td>CMSE</td>
<td>cow’s milk protein sensitive enteropathy</td>
</tr>
<tr>
<td>CTLs</td>
<td>cytotoxic T cells</td>
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<tr>
<td>DBPCEC test</td>
<td>double-blind placebo controlled elimination challenge test</td>
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<tr>
<td>DNAse</td>
<td>deoxyribonuclease</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunoabsorbant assay</td>
</tr>
<tr>
<td>ELISPOTs</td>
<td>enzyme-linked immunoabsorbant spots</td>
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<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>FasL</td>
<td>FasLigand</td>
</tr>
<tr>
<td>FLIP</td>
<td>Fas associated death domain protein –like interleukin 1beta converting enzyme –like inhibitory protein</td>
</tr>
<tr>
<td>GrA</td>
<td>granzyme A</td>
</tr>
<tr>
<td>GrB</td>
<td>granzyme B</td>
</tr>
<tr>
<td>GvHD</td>
<td>graft versus host disease</td>
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<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IELs</td>
<td>intraepithelial lymphocytes</td>
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<tr>
<td>LNHI</td>
<td>lymphonodular hyperplacy</td>
</tr>
<tr>
<td>LP</td>
<td>lamina propria</td>
</tr>
<tr>
<td>LPLs</td>
<td>lamina propria lymphocytes</td>
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<tr>
<td>MADCam-1</td>
<td>mucosal addressin cell adhesion molecule 1</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>Mpr</td>
<td>mannose phosphate receptor</td>
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<tr>
<td>NKs</td>
<td>natural killer cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>PFN</td>
<td>perforin</td>
</tr>
<tr>
<td>PPs</td>
<td>Payer’s patches</td>
</tr>
<tr>
<td>RAST</td>
<td>radioallergoimmunosorbent test</td>
</tr>
<tr>
<td>sFas</td>
<td>soluble Fas</td>
</tr>
<tr>
<td>SPT</td>
<td>skin prick test</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TG2</td>
<td>transglutaminase 2</td>
</tr>
<tr>
<td>Th1</td>
<td>T helper 1</td>
</tr>
<tr>
<td>TIA-1</td>
<td>T cell restricted intracellular antigen type 1</td>
</tr>
<tr>
<td>TIAR</td>
<td>T cell-restricted intracellular antigen type 1 (TIA-1) related antigen</td>
</tr>
<tr>
<td>TJs</td>
<td>tight junctions</td>
</tr>
<tr>
<td>TUNEL</td>
<td>terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine triphosphate nick end labelling</td>
</tr>
<tr>
<td>αβ-TCR</td>
<td>alpha/beta-T cell receptor</td>
</tr>
<tr>
<td>γδ-TCR</td>
<td>gamma/delta-T cell receptor</td>
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List of original papers

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


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1 Introduction

Atopic diseases are becoming increasingly common worldwide, and food hypersensitivities seem to be a part of this rise (Sicherer 2002). This group of conditions includes acute, potentially fatal reactions, and a group of chronic diseases that mainly affect the skin and/or gastrointestinal tract (Sicherer 2003). Adverse reactions to foods are among the causes to consider when evaluating gastrointestinal symptoms and complaints. Gastrointestinal food hypersensitivities consist of a spectrum of disorders that result from adverse immune responses to dietary antigens (Bruijnzeel-Koomen et al. 1995, Johansson et al. 2004).

It has been suggested that intraepithelial lymphocytes (IELs) participate in immune protection, surveillance of the epithelium and induction and maintenance of oral tolerance (Melgar et al. 2004). The granules of cytotoxic IELs and lamina propria (LP) lymphocytes (LPLs) contain two membrane-perturbing proteins, perforin (PFN) and granulysin, and a family of serine proteases known as granzymes (Kummer et al. 1993). The expression of these molecules is significantly induced in activated CD8⁺, CD3⁺ large granular, and gamma/delta (γδ) T cell receptor (TCR) bearing cytotoxic T lymphocytes (CTLs). However, these molecules are normally expressed in human CD3-, CD56⁺ natural killer cells (NKs) (Lichtenheld et al. 1988). CTLs and NKs can destroy their target cells either by PFN/granzyme or Fas/Fas ligand (FasL) pathway, which are the two major known pathways of cell mediated apoptosis (Russell & Ley 2002).

In celiac disease (CD), cytotoxic activation of T-lymphocytes is known to play an important role in the pathogenesis (Green & Jabri 2003). Extensive cytotoxic activation is a characteristic feature as shown by high numbers of duodenal TIA-1, PFN, GrB and Fas/FasL containing IELs (Russell et al. 1993, Oberhuber et al. 1996, Zimmer et al. 1998, Shiner et al. 1998, Ciccocioppo et al. 2000, Ciccocioppo et al. 2002) and both the PFN/granzyme and the Fas/FasL pathways seem to be involved leading to apoptotic enterocyte loss and villous atrophy (Ciccocioppo et al. 2000, Ciccocioppo et al. 2002, Maiuri et al. 2001, Ciccocioppo et al. 2001, Di Sabatino et al. 2001, Strater & Moller 2001).

Cow’s milk protein related non-IgE mediated enteropathies present in two clinically distinct fashions. In infants, the term cow’s milk protein intolerance (CMPI) is generally used to describe a severe form of enteropathy with mucosal damage that is improved by
omitting cow’s milk from the diet. In preschool and school aged children, the term cow’s milk protein sensitive enteropathy, CMSE is recommended. Except for the CMPI cases with malabsorption (Kuitunen et al. 1975, Maluenda et al. 1984, Chung et al. 1999), no evidence of epithelial or villous abnormality on light microscopy has been seen in CMSE (Kokkonen et al. 1999, Kokkonen et al. 2001). The prevalence of CMSE is estimated to be about 2-3% in preschool and school aged children (Kokkonen et al. 2001, Kokkonen et al. 2004). The diagnosis is based on the open or blinded elimination/challenge test, as endoscopic and laboratory findings are often non-specific. Both endoscopic and histological examinations can show non-specific patchy or segmental mild mucosal abnormalities in various parts of gastrointestinal tract including lymphonodular hyperplasia. The accumulation of IELs and γδ⁺-TCRs (Kokkonen et al. 1999, Kokkonen et al. 2000) is a feature shared with CD.

This study was designed to investigate the presence of cytotoxic granule containing CTLs and NK cells in the gastro-duodenal mucosa of CMSE patients, and to determine whether cytotoxic granule proteins can be found in the serum of CMSE and CD patients. The pathological significance of cytotoxicity was assessed by analysing the association with apoptosis and proliferation rates. In addition, these markers were assessed as possible diagnostic markers of CMSE.
2 Review of the literature

2.1 Fetal development of the intestine

2.1.1 Ontogeny of the gastrointestinal tract

Gastrulation, the determination of the embryonal axes with three germ layers is initiated in the 3rd week of gestation. The process is very similar in all vertebrate species (de Santa Barbara et al. 2003). Endoderm is the precursor for the epithelial lining of the gastrointestinal tract, mesoderm forms the smooth muscle layers and ectoderm gives rise to the enteric nervous system. The two major steps in development of the gastrointestinal tract are the formation of the gut tube and formation of the individual organs with their specialized cell types. (Montgomery et al. 1999) By 13 weeks gestation, intestinal organogenesis is complete (Moxey & Trier 1978) as well as the development of the small and large intestine. A high rate of fetal swallowing, detectable from weeks 16-17, is critical for further development (El-Haddad et al. 2004). The longitudinal growth of intestine is accelerated in the last 15 weeks of gestation, leading to a mean length at birth of 275cm, compared with an average length of 575cm at the age of 20 (Weaver et al. 1991).

2.1.2 Mucosal remodelling and villous formation

Beginning at 9-10 weeks and proceeding in a cranial-caudal direction, crypt stem cells produce progenitors which appear undifferentiated in the crypt but eventually develop into four cell types when migrating to the crypt-villus junction: enterocytes, enteroendocrine cells, Paneth cells, and goblet cells. The further development and homeostasis of the mucosa (Figure 1) requires tight control and balance of proliferation, differentiation, migration and apoptosis. (de Santa Barbara et al. 2003) By the 22nd gestational week, the epithelial cells resemble those of the adult intestine (Montgomery et
Lysosomal enzymes and lysosomal activity in human fetal intestine have been reported to begin at weeks 9 to 10 (Antonowicz et al. 1974). At 17-20 gestational weeks, epithelial glucose transport is established (Malo & Berteloot 1987).

2.1.3 Appearing of immunocytes

The number of IELs is 2-5 times lower in 18-22 week foetuses than in older children (Spencer et al. 1989a) and only few LPLs are found. Only approximately 50% of IELs express CD4 or CD8 antigen. (Spencer et al. 1986) Immunologically important specialized microvillus epithelial cells - M cells - are present by 17 weeks, overlying lymphoid follicles of maturing Payer’s patches (PPs) (Trier & Moxey 1979) PPs are detectable by 20 weeks, but they lack germinal centres, which only develop after birth as a result of antigen challenge (Brandtzaeg et al. 1991).

Fig. 1. A light microscopic view of two mucosal villi in the descending duodenum; immunohistochemical CD3 (T-lymphocyte) staining; a biopsy sample of CMSE patient; original magnification x 100. Epithelium (EP), villous lamina propria (VLP), lamina propria lymphocytes (LPLs), crypt (C), muscularis mucosae (MM), villus-crypt (V-C) border, intraepithelial lymphocytes (IELs), epithelial cells (ECs), goblet cell (GC). Out of an optimally cutted villus, the upper (U), middle (M) and lower (L) thirds are demonstrated.
2.2 The main functions of the gastrointestinal tract

2.2.1 Digestion

To fulfil the digestive function, the adult gut has a surface area of 400 m², which is lined by a single 30μm thick layer of enterocytes, each interconnected to adjacent cells by desmosomes, adherens and tight junctions. The whole surface area is shed and replaced every 2-3 days. (MacDonald 2003) Ingested proteins are absorbed from the gastrointestinal tract either as intact proteins by the paracellular pathway (van Niel et al. 2001) or as peptide fragments degraded by lysosomal proteins into smaller peptides (transcellular pathway). Only a small proportion of proteins is transported intact by the paracellular pathway, but can result in antigen-specific immune responses. In addition to intercellular tight junctions (TJs) and cadherins, which serve as the main barrier to macromolecules (Madara 1990, Higgins et al. 1998), transcellular and paracellular fluxes are tightly controlled by membrane pumps and ion channels, adapting permeability to physiological needs. (Baumgart & Dignass 2002) Infants, who have a more permeable gut barrier and therefore increased absorption compared to older people (Walker 1979), are at greater risk of food hypersensitivities. Older children with cystic fibrosis and defects in enzymatic antigen degradation have increased absorbance of allergenic macromolecules and have been shown to have an increased incidence of cow’s milk allergy (CMA) (Lucarelli et al. 1994). The same phenomenon is seen in patients with inflammatory bowel diseases (Levo et al. 1986). The molecular size detected by IgE antibodies is about 20 000-100 000 kDa, whereas cell mediated response recognizes smaller (5 000-30 000 kDa) molecules, which can explain the delayed onset of non-IgE-mediated symptoms in the lower gastrointestinal tract.

2.2.2 Oral tolerance

Whilst allowing the transfer of nutrients from the intestinal lumen to the systemic circulation, the mucosa also protects against pathogens by the induction of immune responses. A down-regulation of immune responses to non-harmful ingested antigens is termed oral tolerance (Shah & Walker 2002); mature lymph node lymphocytes become hyporesponsive after oral antigen administration (Strobel 2002).

In normal individuals with tolerance, systemic and secretory food-specific IgA antibodies are generally absent, indicating that mucosal IgA production is regulated in a way similar to that of systemic immunity (Strobel & Mowat 1998). However, mucosal IgA response to foreign antigens remains active (Mestecky & McGhee 1987). On the other hand, serum IgA deficiency is associated with CD and other food hypersensitivities (Cataldo et al. 1998, Ludviksson et al. 1992) including CMSE (Kokkonen et al. 2001, Kokkonen et al. 2001).

The absorbed food antigens interact with mucosal T and B cells either directly or through antigen-presenting cells (APCs): macrophages, dendritic cells, or M cells. T cell
recognition of antigen requires TCR and the major histocompatibility complex (MHC) molecules (class I and II) of APCs. Activated T and B cells of lymphoid follicles migrate first via the lymphatic system and then via the circulation to any of several target organs including the gastrointestinal tract, the respiratory system, the skin, and the central nervous system, the process which is referred to as “homing”. If tolerance is not achieved, T and B cells will activate at a homing site upon contact with their specific food antigen and release their cytokines, vasoactive peptides and antibodies giving rise to an inflammatory reaction in the affected organ and result in the clinical manifestations of food hypersensitivity. (Sabra et al. 2003).

To assess the possible development of oral tolerance in cases of food hypersensitivities, periodic challenges with food allergen can be considered. This approach is contraindicated where there is a recent history of severe symptoms or a strong possibility of acute, IgE-mediated reactions. However, as most IgE-mediated food allergies of childhood improve over time, cautious testing - starting on skin - is recommended in older children. In non-IgE food allergies, reassessment can be tried once or twice a year, starting with a very small dose. Multiple low doses are more likely to generate regulatory cells (Melamed et al. 1996), as there is evidence in a mouse model that single administration of high doses of antigen induces suppression of virtually all responses including tolerance by direct inactivation of T cells (Melamed & Friedman 1994, Garside et al. 1995).

2.3 Food hypersensitivity

According to currently accepted international guidelines, the term hypersensitivity describes objectively reproducible symptoms or signs initiated by exposure to a defined stimulus at a dose tolerated by normal persons (Johansson et al. 2004). In food hypersensitivity, the term food allergy is appropriate when specific immunologic mechanisms have been demonstrated. Food allergy can be either antibody-mediated or cell-mediated, or in some cases both may be involved. If IgE is involved in the reaction, the term atopic food allergy is appropriate. If immunological mechanisms other than IgE are dominantly involved, term non-IgE-mediated food allergy should be used. All other reactions should be regarded to as non-allergic food hypersensitivity (Bruijnzeel-Koomen et al. 1995, Ortolani et al. 1999). Because of secondary hyper-reactivity, the symptoms can also be induced or accelerated by non-immunological factors such as emotions or exercise (Johansson et al. 2004). Table 1 categorizes the broad spectrum of allergic and non-allergic food hypersensitivities.

The etiology of allergy is unclear. Genes linked to the expression of cytokines, chemokines, antibodies and TCRs might be partly responsible (Barnes 2000) together with environmental factors, such as an overly hygienic environment (Yazdanbakhsh et al. 2002).
2.3.1 Mechanisms of allergy

Enhanced immune mediated reactivity may be mediated by any, or a combination of, the four basic types of immunologic reactions outlined by Gell and Coombs. Type I or IgE mediated hypersensitivity leads to immediate symptoms, such as urticaria, angio-oedema and/or other anaphylactic reaction. In type II (cytotoxic) reactions, the antigen binds to the cell surface and the presence of antibodies (IgG, IgM, or IgA) disrupts the membrane leading to cell death. In type III (Arthus-type) reactions, antigen-antibody-complement immune complexes (IgG, IgM, IgA and IgE antibodies) get trapped in small blood vessels or renal glomeruli. Finally, the type IV (delayed) reactions, which are mediated by sensitized T lymphocytes. Type I reactions are best understood, and are often referred to as the most common and classic allergic reactions. The three other types, collectively described as non-IgE-mediated allergy, are more difficult to investigate and hence less well understood. In an individual, several types of immune responses may be activated, though IgE-mediated reactions are more usually measured. For example, in infants with measurable IgE reactivity undergoing endoscopic examinations, local cell mediated reactivity seen as lymphoid nodular hyperplasia (LNH) may be demonstrated in most cases. (Murch S 2004)

Secretory IgA is considered as one of the crucial elements in the protection of small intestinal mucosa against invading antigenic material. Maturational delay in IgA response has been found to predispose to the development of CMA in infants and small children (Tiller & Buckley 1978). In a population survey from Iceland, more allergic sensitization was seen in subjects with an IgA level at the lower end of the normal range (Ludviksson et al. 1992). In older children with CMP1/CMSE there are controversial results in milk specific IgG and IgA blood levels found between patient and control groups (Kokkonen et al. 2002, Motrich et al. 2003). But with total IgA, both pre- and post-challenge blood levels have been shown to be higher in CMA compared to controls. Interestingly, regardless of the challenge result, total IgA concentration increased during the challenge in all the infants. In infants reacting to the cow’s milk challenge, higher pre-challenge levels of fecal IgA were found. (Saarinen et al. 2002) Children at 10-11 years of age reacting in an open or blinded challenge with low-lactose milk showed significantly lower IgA-class antibodies to milk and its fractions than the non-symptomatic controls (Kokkonen et al. 2004). In non-IgE mediated CMP1 with mucosal damage, IgA- and IgM-containing cells were reported to be markedly increased during cow’s milk exposure (Savilahti 2000). But when healthy infants were put on a cow’s milk elimination diet, the numbers of immunoglobulin secreting cells of IgM and IgG classes measured by ELISPOT assay were decreased (Kaila 1993).

2.3.2 Atopic, IgE-mediated food allergy

IgE-mediated, acute onset food allergies affect one or more target organs: the skin (urticaria, angio-oedema), respiratory tract (rhinitis, asthma), gastrointestinal tract (pain, flatulence, emesis, and diarrhea), and/or the cardiovascular system (anaphylactic shock). (Sicherer 2002) The prevalence of IgE-mediated food allergy in infants varies from 6% to
8%, being 1% to 2% in adults. In contrast to adults, atopic food allergy in childhood, (often a part of the “allergic march”), resolves in more than 85% of children, especially those with hypersensitivity to cow’s milk and egg. (Thong & Hourihane 2004) The reported incidence of IgE-mediated CMA varies from 0.1 to 7.5%, being 1.9-2.8% among infants under 2 years old, but its incidence falls to approximately 0.3% in children older than 3 years (Host 1994, Host & Halken 1990, Bock 1987, Schrander et al. 1993, Hill 1986, Saarinen et al. 2000). However, CMA may also occur in adults. Björnsson et al (1996) found a prevalence of 1% of probable IgE-mediated CMA in adults in Sweden. The non-antibody active IgE molecules have no allergy-related biological activity, thus, IgE-mediated food allergy cannot be defined on the basis of an increased level of total IgE, or presumed from the presence of IgE on a cell surface. Food-specific IgG-antibodies of serum are not of clinical importance but merely indicate previous exposure to the food. (Husby 2000)

2.3.3 Non-IgE-mediated food allergy

Subacute and chronic non-IgE-mediated food allergies typically present with gastrointestinal (Sampson et al. 2001) or skin symptoms (Sicherer & Sampson 1999). CD, CMSE, dietary protein enterocolitis, and proctitis are the most common clinical manifestations. It seems possible that during the chronic phase of an originally IgE-initiated allergic inflammation, the inflammatory reaction causing the symptoms is dominated by allergen-specific lymphocytes. This is often the case in school-aged children with CMSE (Kokkonen et al. 2001). Dermatitis herpetiformis is a skin manifestation that is sometimes associated with CD (Bodvarsson et al. 1993). Atopic dermatitis is a common manifestation of food hypersensitivity triggered by both IgE and T cell-mediated mechanisms (Sabra et al. 2003). Children with atopic dermatitis that do not respond to several months of routine therapy are thought to benefit from evaluation for possible food hypersensitivity (Burks et al. 1988).

2.3.4 Differential diagnosis

When diagnosing a patient with prolonged gastrointestinal symptoms and/or signs, the clinician has to take many conditions into consideration including: constipation, irritable bowel syndrome, inflammatory bowel diseases, esophagitis, gastritis, peptic ulcer disease, post-infectious enteropathy, autoimmune enteropathy, malnutrition, tropical sprue, infections such as Helicobacter pylori, parasites, malignancy, endocrinological and metabolic diseases, microvillus inclusion disease etc. Food intolerance reactions may be confused with allergic and immunologically mediated reactions, such as toxic effects (bacterial contamination, food-borne toxins, and food additives). Intolerance to enzyme deficiency (lactase and other disaccharide deficiencies, tyrosinemia and other inborn metabolic abnormalities) symptoms due to pharmacological properties of food (e.g. tyramine and histamine in food stuffs) can also produce similar symptoms. Finally, temporary functional, immune-response mediated or morphological abnormalities of
small intestinal mucosa due to various reasons including hormonal, nervous, changes in luminal homeostasis, infectious and other reasons challenge the true diagnosis of immune mediated food hypersensitivity. (Murch S 2004)
<table>
<thead>
<tr>
<th>Food hypersensitivity</th>
<th>Age group</th>
<th>Prognosis</th>
<th>Clinical picture</th>
<th>Cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgE-mediated food allergy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral allergy syndrome</td>
<td>children/adults</td>
<td>waxes/wanes</td>
<td>oral pruritus, edema</td>
<td>fruits or vegetables</td>
</tr>
<tr>
<td>Acute gastrointestinal hypersensitivity</td>
<td>any, mostly infants</td>
<td>variable</td>
<td>nausea, emesis, pain, diarrhea</td>
<td>milk, egg, wheat, soy, peanut, tree nuts, seafood</td>
</tr>
<tr>
<td>Allergic eosinophilic gastro-enteropathy</td>
<td>any</td>
<td>variable</td>
<td>dysphagia, pain, ascites, weight loss, reflux, oedema, obstruction</td>
<td>multiple foods</td>
</tr>
<tr>
<td>Non-IgE-mediated food allergy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Idiopathic eosinophilic gastro-enteropathy</td>
<td>any</td>
<td>variable</td>
<td>dysphagia, pain, ascites, weight loss, reflux, oedema, obstruction</td>
<td>unknown</td>
</tr>
<tr>
<td>Dietary protein enteropathy</td>
<td>infants/children</td>
<td>resolves</td>
<td>malabsorption, oedema, emesis, poor growth</td>
<td>cow’s milk</td>
</tr>
<tr>
<td>Dietary protein enterocolitis</td>
<td>infants</td>
<td>resolves in 1-3 years</td>
<td>emesis, diarrhea, poor growth, lethargy, hypotension</td>
<td>cow’s milk, soy, grains</td>
</tr>
<tr>
<td>Dietary protein proctitis</td>
<td>infants</td>
<td>resolves in 1 year</td>
<td>mucous, bloody stools</td>
<td>breast milk with maternal cow milk ingestion, cow milk</td>
</tr>
<tr>
<td>Celiac disease</td>
<td>any</td>
<td>permanent</td>
<td>flatulence, pain, diarrhea, malabsorption</td>
<td>gluten</td>
</tr>
<tr>
<td>Food hypersensitivity</td>
<td>Age group</td>
<td>Prognosis</td>
<td>Clinical picture</td>
<td>Cause</td>
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<td>-----------------------</td>
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</tr>
<tr>
<td><strong>Adverse, non-immunological reactions to foods</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Constipation</td>
<td>any</td>
<td>resolves</td>
<td>abdominal pain and distension</td>
<td>low-fibre diet</td>
</tr>
<tr>
<td>Lactose intolerance</td>
<td>any</td>
<td>mostly permanent</td>
<td>flatulence, pain</td>
<td>lactase deficiency</td>
</tr>
<tr>
<td>Post-infectious enteropathy</td>
<td>any</td>
<td>resolves</td>
<td>diarrhea/constipation, flatulence</td>
<td>bacterial, virus, parasitic</td>
</tr>
<tr>
<td>Gastro-esophageal reflux</td>
<td>any</td>
<td>variable</td>
<td>eructation, rumination</td>
<td>dosage and stature dependent, multiple foods</td>
</tr>
<tr>
<td>Pharmacological effects of food constituents</td>
<td>any</td>
<td>dose dependent</td>
<td>cutaneous flashing</td>
<td>caffeine, histamine, tyramine</td>
</tr>
<tr>
<td>Malabsorption</td>
<td>any, mostly middle age or older</td>
<td>mostly permanent</td>
<td>pain, various abdominal symptoms</td>
<td>gallbladder disease, pancreatic insufficiency</td>
</tr>
<tr>
<td>Metabolic disorders</td>
<td>variable</td>
<td>permanent</td>
<td>various</td>
<td>eg. galactosaemia</td>
</tr>
<tr>
<td>Toxins</td>
<td>any</td>
<td>resolves</td>
<td>acute onset, various gastrointestinal and systemic symptoms</td>
<td>bacterial, scombroid fish poisoning</td>
</tr>
<tr>
<td>Neurological</td>
<td>any</td>
<td>dose dependent</td>
<td>rhinitis, facial flush</td>
<td>spices</td>
</tr>
<tr>
<td>Colic</td>
<td>young infants</td>
<td>resolves</td>
<td>pain</td>
<td>unknown</td>
</tr>
<tr>
<td>Psychological</td>
<td>any, adolescents</td>
<td>curable</td>
<td>swallowing problems</td>
<td>mental problems</td>
</tr>
</tbody>
</table>

Modified from reviews of Sicherer (Sicherer 2002) and Sampson (Sampson 2003).
2.3.5 **Cow’s milk protein sensitive enteropathy**

### 2.3.5.1 History

Several authors in the 1960’s described a syndrome of mucosal malabsorption due to mild or moderate abnormalities in the small intestinal villous structure appearing dominantly in infants within the first six months of life without cereals in the diet. The symptoms included chronic diarrhea, weight loss, growth failure, and severe nutrient malabsorption. In the beginning, the disease was usually referred to as CMPI, as the syndrome was cured by the elimination of cow’s milk. The children had a varying degree of villous atrophy with crypt hyperplasia and inflammation in jejunal biopsy specimens (Kuitunen *et al.* 1975, Walker-Smith *et al.* 1978, Verkasalo *et al.* 1981). Phillips and co-workers also found increased densities of IELs in untreated cases compared to the controls, the densities decreasing during an elimination diet and increasing during a milk challenge along with clinical and morphological relapse. However, the reaction was markedly different from the response to gluten in celiac patients. Most patients with CMPI had also secondary lactose malabsorption (Powell 1978). Unlike children with CD, the mucosal lesions in this disease were considered to be more widespread, starting in the stomach and continuing throughout the gastrointestinal tract. The lesions resolved during a milk elimination diet (Simila *et al.* 1978). This severe form of disease almost vanished from Finland during the 1990’s, and similar trends have been seen in other European countries (Savilahti 2000).

In the late 1970’s, there were reports of toddlers and preschool-aged children with a non-IgE-mediated enteropathy without villous shortening or crypt hyperplasia but with increased density of IELs and sometimes LP eosinophils (Kokkonen *et al.* 1981). These children suffered from vague complaints such as constipation and diarrhoea and/or anaemia but usually showed no immediate symptoms. The symptoms subsided after elimination of cow’s milk from the diet.

In 1999, Kokkonen and co-workers presented a small series of school-aged children with similar symptoms and histological changes but with lymphoid nodular hyperplasia (LNH) of the duodenal bulb found on endoscopy. Later series showed that the routine histological findings in the biopsy samples from the duodenum were minimal, but the number of lymphoid nodules (Kokkonen *et al.* 1999) and ^-TCRs of CD3 IELs were increased (Kokkonen *et al.* 2000). The children had no signs of malabsorption, but an increased incidence of lactose intolerance and their growth was retarded. Both these signs were considered to be a functional disturbance of the epithelial cell layer of the small intestine. The mild mucosal lesions were shown to affect the gastrointestinal tract from mouth to anus, and likely to be patchy. (Kokkonen *et al.* 2001)

In the paediatric literature there is a view that IgE-mediated CMA of infancy remits by the age of 2-5 years in the majority of cases (Sicherer 2002, Sicherer 2003, Sampson 2003). However, Tikkonen and co-workers (2000) showed that children with previous IgE-mediated CMA have an increased incidence of gastrointestinal complaints after
moderate or high doses of milk or dairy products in their diet, even though they no longer had immediate atopic symptoms after milk exposure. Thus most avoided milk, at least as a drink, the incidence of milk-induced gastrointestinal complaints, apart from lactose intolerance, was found to be up to 3% in a series school-aged children (Tikkanen et al. 2000). In a Finnish population study of 206 young adults, the prevalence of milk hypersensitivity was estimated to be 3-6% (Pelto et al. 1999).

Based on the above studies, it was concluded that even in school-aged children, there might be a T cell mediated enteropathy, which might resemble CD on one hand and non-IgE-mediated hypersensitivity of small infants (CMPI) on the other hand. We may conclude that cow’s milk protein related non-IgE mediated enteropathies exist in two clinically different entities. In infants, the term CMPI is generally used to describe the severe form of enteropathy with mucosal damage that is improved by omitting cow’s milk from the diet. In preschool- and school aged children, the term CMSE is recommended.

2.3.5.2 Clinical presentation

In Denmark, a prospective cohort study of 1749 newborns showed that by the age of 3, 6.7% of children had symptoms suggestive of IgE or non-IgE-mediated cow’s milk hypersensitivity, but the diagnosis was proven only in 2.2% (Host & Halken 1990). The prevalence of non-IgE CMSE in school-aged children is estimated to be about 2-3% (Kokkonen et al. 2001, Kokkonen et al. 2004). The delayed gastrointestinal symptoms may include diarrhea and/or constipation and recurrent abdominal pain. The gastrointestinal response varies, possibly because of immunological heterogeneity. Unlike CMPI, the symptoms usually take some time to develop, and the relationship between milk ingestion and symptoms is not easily recognized. There are rarely any physical signs, except weight loss in some cases with prolonged symptoms and a very poor caloric intake.

2.3.5.3 Pathogenesis

Milk allergy seems to be largely directed against three proteins found in milk: alfa-lactalbumin, beta-lactoglobulin, and caseins. Allergy tests usually only include caseins. Among caseins, alfa(s) 1-casein is a major allergen in milk. In highly atopic, milk allergic children it has been found that every protein tested reacts with IgE antibodies in the patient’s serum. However, the role of milk proteins in the allergic reactions is not yet clearly understood. (Savilahti & Kuitunen 1992, Wal 2004)

No information about the possible effects of cytotoxic lymphocyte activation in CMSE has been available. Unlike CD, CMSE is not associated with malignancy (Walker-Smith 1992). Most CMSE patients show no evidence of epithelial or villous abnormality at the light microscopic level, but the number of IELs may be slightly increased (Phillips et al. 1979).
The exclusive presence of CD1d, a cell surface glycoprotein with non-MHC encoded antigen-presenting molecules, in the duodenal LP of the patients with non-IgE-mediated cow’s milk hypersensitivity suggests that these molecules are involved in the pathogenesis of food allergies (Ulanova et al. 2000).

2.3.5.4 Clinical tests

According to the ESPGHAN committee chaired by professor Erkki Savilahti, a validated elimination and challenge test with the suspected food stuff is the diagnostic test of choice. The report refers to a double-blind placebo-controlled food challenge (DBPCFC) test. This test has been validated in small children with rapid reactions to minimal doses that appear over hours or up to 7 days. However, we lack a test for diagnosing cases reacting to larger doses and over longer time periods. Assuming we have a celiac disease-like enteropathy with a local cell mediated immune response produced by high doses of cow’s milk (e.g. 50 cc), there would need to be several weeks of elimination and weeks to months of challenge. Practically, there is no such test. Clinicians rely on a 2 to 4 week elimination diet and a response to an oral challenge test with written diaries. In some cases, endoscopy and biopsies of the small intestine or colon may help in determining the diagnosis. This is true especially where there are questions concerning differential diagnosis and/or a child continues to have gastrointestinal symptoms.

If there is a history of acute reactions related to milk or suspicion of IgE-mediated mechanisms, the possibility of severe reactions should always be considered when antigens are re-introduced (David 1984). These patients are challenged carefully in hospital. In Finland, the DBPCFC test performed on the ward is nowadays considered to be an expensive and unpractical method of confirming milk-related symptoms (Duodecim 2004). Single-blind food challenge and open food challenge are the most common techniques but with the open test, the placebo effect may confuse the symptom diary. Also, with the DBPCFC test, the rate of false negative diagnosis is about 3% because the milk challenge is of insufficient duration (Caffarelli & Petroccione 2001).

A strict elimination diet is the only curative treatment. Periodic reassessment with cow’s milk is recommended once or twice a year, starting with a very small dose.

2.3.5.5 Laboratory tests

If a patient has acute skin reactions associated with ingestion and/or skin contact with cow’s milk, skin prick tests (SPTs) are frequently used to screen for possible IgE mechanisms. The negative predictive value of the skin prick test is more than 95%, but a positive SPT merely suggestive of a clinical food allergy. When 239 infants were challenged with cow’s milk, a positive SPT reaction to cow’s milk (≥3mm) was seen in 61% of children known to have CMA, but the test had a false positive rate of 24% (Saarinen et al. 2001). In older children, the accuracy of SPT is even worse, this is why
the Finnish Medical Society Duodecim considers the test to be of use only in infants with rapid-onset skin symptoms related to cow’s milk exposure (Duodecim 2004). Food hypersensitivity is more likely in children with severe, treatment-resistant atopic dermatitis, than those with intermittent or mild dermatitis (Sicherer & Sampson 1999, Burks et al. 1988, Sampson & McCaskill 1985). In food-responsive atopic dermatitis, the skin may be targeted by food-specific T cells expressing skin homing antigens (Abernathy-Carver et al. 1995). The “atopy patch test” (APT) lasting one to three days is sometimes used in combination with SPTs to test children with delayed skin manifestations (Niggemann et al. 2000). However, diagnosis can be difficult because of frequent false positive results due to unspecific skin irritation (Saarinen et al. 2001, Vanto et al. 1999). A standardised and validated investigation protocol has not yet been determined for Finland (Duodecim 2004).

The radioallergosorbent test (RAST) detects milk-specific IgE antibodies, and is one method of detecting IgE-mediated mechanisms (Duodecim 2004). RAST results correlate with SPT results (Saarinen et al. 2001, Vanto et al. 1999), therefore both tests are seldom needed. The negative predictive value is about 95%, but in chronic cases the specificity of a positive RAST result is only 50%, since positive titres can be found for a long time after clinical improvement (Sampson 1999). In older children, only high titres should be considered to be true positives.

2.3.5.6 Endoscopy

There seem to be no unique endoscopic findings in food hypersensitivity. LNH is the most common finding in both infants with atopic food allergy and school-aged children with delayed reactivity. Endoscopy may also be entirely normal. In a few food allergy case series, LNH has been seen in the duodenal bulb in 30 – 75% of cases, in the colon in 50-80% and in the terminal ileum in 70-90% of cases. A patchy appearance and a distal distribution are typical features of LNH related to food hypersensitivity. In the foregut, the nodules are most often seen in the bulb, lesions continuing to the descending duodenum in only a quarter of cases. In the terminal ileum, LNH is usually restricted to an area 10 – 15 cm proximal to the valve. If massed just above the valve it usually becomes nodular and disappears entirely when proceeding proximally to the ileum. Colonic lesions are usually in the transverse colon but clusters of nodules may be spread throughout the colon or concentrated in any segment. (Kokkonen et al. 1999, Kokkonen et al. 2004, Murch S 2004)

Aphthous ulcers, erosions, gastritis, duodenitis and different types of colitis are additional macroscopic findings in food hypersensitivity (Murch S 2004). As it is usually non-specific, endoscopic examination is indicated only cases with vague and prolonged gastrointestinal symptoms that remain undiagnosed after an extended open elimination and challenge test. There is a clear need for a reliable diagnostic test. Detecting changes in cytotoxic activity during an antigen challenge might be a reliable means of quantifying the immunological activity in food allergies.
2.3.6 Celiac disease

The first report of a gastrointestinal condition resembling CD is thought to be that of Arataeus of Cappadocia in the second century AD. In 1950, the connection between the ingestion of certain cereals and the onset of gastrointestinal symptoms typical of CD was established by a doctoral thesis in the University of Utrecht, Holland by Willem-Karel Dicke, who was also the pioneer of the gluten free diet (van Berge-Henegouwen & Mulder 1993). CD was thought to be a disease with a consistent clinical presentation, affecting mainly infants. It is now recognised that there can be later presentations in older children and adults with less typical symptoms.

2.3.6.1 Clinical presentation

Epidemiological studies have revealed CD to be one of the most frequent genetic diseases in humans. Serologic screening studies have shown the prevalence in adults to be 1 in 266. (Fasano & Catassi 2001) During the past three decades, the clinical definition has been expanded to include milder forms, thus resulting in an upward shift in the age at diagnosis (Maki et al. 1988). In Northern Ostrobothnia, Finland, the prevalence of CD among school-aged children was estimated to be at least 1 case in 99 children based on a variety of tests including serum screening, human leukocyte antigen (HLA) typing and small-bowel histology (Maki et al. 2003).

Diarrhea, the classic symptom in CD, is usually due to a progression of mucosal changes found in distal duodenum (RUBIN 1960). However, abdominal pain, vomiting, irritability and constipation are common (Green & Jabri 2003, Green et al. 2001). Most severe cases in infants and young children present with a failure to thrive (Lowichik & Book 2003). Older children often present with extraintestinal manifestations, such as anaemia, short stature (Maki et al. 1988), dermatitis herpetiformis (Bodvarsson et al. 1993) or neurological symptoms (Molteni et al. 1988, Hadjivassiliou et al. 1998, Alaedini et al. 2002).

The presence or absence of gastrointestinal symptoms can be used to classify the disease into two categories: Symptomatic, active or classic CD with diarrhea and asymptomatic or silent CD where gastrointestinal symptoms are mild or absent. In the Finnish population, about one third of adult patients have been shown to belong to the latter group (Collin et al. 1997). In children, the disease can be missed if the diagnosis relies only on classical symptoms such as diarrhea and short stature (Maki et al. 2003, Rossi et al. 1993).

CD is associated with an increased rate of osteoporosis both in children (Scotta et al. 1997) and adults (Valdimarsson et al. 1994). Infertility (Sher & Mayberry 1994) and malignancy, especially lymphomas (Holmes et al. 1989) are also more common. Whether autoimmune diseases could be prevented by the early diagnosis and treatment of CD, is a topic of intensive research at the moment. For example, reduced levels of diabetes- and thyroid-related serum antibodies have been seen in children with CD on a gluten-free diet (Ventura et al. 2000). However, a Finnish study demonstrated that patients on a strict
A gluten-free diet had no greater mortality than those of the general population (Collin et al. 1994).

### 2.3.6.2 Pathogenesis

CD is a cell mediated chronic inflammatory disease (Sollid 2002), which is classified as a food-allergic disorder (Johansson et al. 2004, Farrell & Kelly 2002). Changes in intestinal permeability together with an inappropriate immune response against gluten found in wheat, rye and barley, lead to a complex inflammatory disorder in which the environment and several genes are contributory factors. HLA is the most important single genetic factor HLA-DQ2 being found in most, and HLA-DQ8 in a minority of CD patients.

CD is due to an abnormal CD4 T helper cell type 1 (Th1) response. The immune response takes place in two compartments, the LP and the epithelium. In the LP, isolated gluten-specific CD4+ T cells recognize several gluten epitopes only in the context of DQ2 or DQ8. CD4+ T recognise most epitopes in a deamidated form – in other words, some specific glutamine residues have been converted into glutamic acids mediated by the enzyme transglutaminase 2 (TG2). Gluten-specific T cells might help TG2-specific B cells by linked recognition of TG2-gluten-peptide complexes. This could explain how the exposure to a foreign antigen leads to the formation of autoantibodies. The role of CD8+ IELs in pathogenesis is controversial. Unlike in the LP, no anti-gliadin restricted IELs have been found. (Sollid 2002). Still, the pathogenic role of epithelial lymphocytosis has been demonstrated by the manifestation of abnormal IEL expansion in CD-related lymphoma (Cellier et al. 2000).

Also, recent evidence suggests that the theory presented in the 1960’s, that gliadins or their metabolites may directly injure the intestinal mucosa, may have some validity. Up-regulation of mucosal HLA-DR and intercellular adhesion molecule-1 (ICAM-1) within 2h of an *in vitro* exposure to gliadin suggest an early effect which may not be immune-mediated (Picarelli et al. 1996). Another sign of a putative non-specific immune mechanism is the consistent presence of γδ-TCR+ T cells both in the LP and epithelium of CD children even after withdrawal of gluten (Savilahti et al. 1990).

### 2.3.6.3 Serological tests

The specificity of IgA-antibodies against endomysium is almost 100% and the performance of tissue transglutaminase antibodies may be even better (Green & Jabri 2003, Green & Jabri 2003). In 1.7-2.6%, CD is associated with a selective IgA deficiency. Therefore total serum IgA needs to be included in screening (Cataldo et al. 1998) and followed by measurement of IgG endomysium or transglutaminase antibodies, which are usually increased with these patients (Cataldo et al. 2000).

The inclusion of HLA typing is useful if, for example, a patient is already on a gluten-free diet or serological tests and histology are negative. People who have neither a HLA-DQ2 or HLA-DQ8 haplotype are unlikely to have CD, since more than 98% of the CD patients express one of these class II major histocompatibility complexes (Kaukinen et al. 1994).
However, as HLA-DQ2 and HLA-DQ8 are found in about 20% of the population, they do not have a useful positive predictive value (Pecsi 2000).

In addition to screening patients with suspected symptoms, serological tests have an important role in monitoring the effect of a gluten-free diet, which is the only effective treatment for CD at present.

2.3.6.4 Small bowel biopsy

According to current recommendations, the diagnosis of CD is based on duodenal biopsy. An increased number of IELs, shortening of villi (villous atrophy) and lengthening of crypts (crypt hyperplasia) especially in the bulb mucosa are the main histological findings (Green & Jabri 2003, Perera et al. 1975). A striking increase in $\gamma\delta$-TCR bearing IELs is an additional diagnostic indicator for CD (Savilahti et al. 1990). Misinterpretation of slides is often due to poor sample quality and tangential sectioning. For correct diagnosis, in addition to a sample taken from the bulb, at least three tissue specimens of adequate size and correct orientation should be taken from the duodenum (Shidrawi et al. 1994).

A major challenge in diagnosis is the interpretation of mild or borderline cases. Before actual villous atrophy is established, crypts become shallower and the crypt/villus ratio decreases from 1:3 to 1:2. Atrophic changes are often patchy and may be present only in the bulb, hence an adequate number of biopsies need to be taken (Vogelsang et al. 2001, Bonamico et al. 2004). IEL counting is recommended in borderline cases where the histology is difficult to interpret (Jarvinen et al. 2003b). The previous IEL index threshold of 40/100 (Ferguson & Murray 1971) has been reduced to 25/100 to include milder cases (Hayat et al. 2002, Mahadeva et al. 2002, Veress et al. 2004). Villus tip analysis, which means counting IEL score per 20 enterocytes in the villus tip region, may help in distinguishing early CD from non-specific changes, thus providing a valuable tool in routine practice, especially when borderline findings are involved. The sensitivity of villus tip analysis appears to be similar to the counting of gamma delta receptor positive T cells, but it does not require frozen biopsy samples. (Jarvinen et al. 2004) However, an increase in IELs seems to be a non-specific finding in the majority of cases (Mahadeva et al. 2002, Kakar et al. 2003a). Intraepithelial lymphocytosis (with an IEL index of 40/100) in small bowel biopsy samples with normal mucosal architecture was related to CD only in 9% of adult cases (Kakar et al. 2003b).

2.4 Small intestinal immune system and food allergens

The organized lymphoid tissue of the small intestine was first described in humans by de Payer in 1687. Structurally, PPs are organized areas of lymphoid tissue in the mucosa of the small intestine. It seems that PPs are the inductive site of mucosal immunity and the LP and epithelium are the effector sites, but there is a growing realization that mucosal immune responses can occur in the absence of PPs and that antigen sampling may also occur in the lamina propria. (MacDonald 2003)
2.4.1 Intraepithelial lymphocytes

IELs are the first immune system cells to encounter pathogens and allergens on the intestinal epithelial surface. Since each lymphocyte recognizes only a single antigen, a huge number of circulating lymphocytes are produced in bone marrow and thymus every day. Increasing evidence suggests that some IELs may arise extrathymically. IELs are present on the other side of the basement membrane from the lamina propria, between the intestinal epithelial cells, the density being approximately 10-20 IELs per 100 villous enterocytes in the healthy human small bowel (Ferguson 1977). IELs exhibit various cytotoxic activities including alloreactive and antigen-specific CTL activity, NK activity and spontaneous cytotoxicity (Beagley & Husband 1998). These functions are required in immune protection, surveillance of the epithelium and induction and maintenance of oral tolerance (Melgar et al. 2004). IELs secrete a variety of cytokines. Subsets of IELs have been shown to act with B cells, to play a role in the maintenance of oral tolerance and to regulate epithelial cell function. (Beagley & Husband 1998)

2.4.1.1 The phenotype of IELs

IELs can be distinguished from their systemic counterparts by their surface composition, as over 70% of small intestinal IELs express CD8 antigen. Compared to systemic T cells, a substantial fraction of IELs are so called large granular lymphocytes differing in morphology, size and sedimentation density (Ferguson 1977, Rudzik & Bienenstock 1974).

The four TCR chain gene families (α, β, γ, δ) have been strongly conserved across 400–500 million years of evolution of the jawed vertebrates (Hayday 2000). Nowadays, three broad categories of IELs are recognised: the subset of γδ-TCR+ IELs and two distinct subsets of αβ-TCR+ IEL, those that express one of the conventional TCR co-receptors, CD4 or CD8αβ, and those that lack co-receptor expression and are double negative. A common feature of all CD8+ IEL subsets is the unique capacity to express a αα-homodimer, which is associated with suppressor/cytotoxic activity. Antigens presented with class II MHC molecules are recognized by CD4+ T cells with their CD3 αβ-TCR whereas CD8+ CTLs and NK cells recognize antigens associated with class I MHC molecules. (Cheroutre 2004, Hayday et al. 2001). In humans, γδ-TCRs are present in greater numbers in intestinal epithelium than in the circulation (Hayday 2000). They do not recognize antigens presented by polymorphic MHC molecules of APCs but are probably primed directly by epithelial cells in situ (Hayday et al. 2001).

2.4.2 Lamina propria lymphocytes

Between the epithelium and the muscular mucosa, is a layer of immunologically important connective tissue, the LP. A large number of LP macrophages, dendritic cells and T cells ensure that antigens crossing the epithelium are processed and presented to LP
CD4+ T cells which, in addition to cell-mediated immunity, help B cells in humoral immunity. (MacDonald 2003)

### 2.4.2.1 Lamina propria T cells

LP T cells are mostly of the helper/inducer phenotype (CD4+) the suppressor/cytotoxic phenotype bearing the αβ-TCR. A minority are CD8+. After antigen stimulation, CD4+ LPLs are derived from T blasts in PPs, which left the circulation with the help of α4β7 integrin binding to mucosal addressin cell adhesion molecule 1 (MAdCAM-1) (Butcher & Picker 1996). LP T cells remain as resting memory cells until they are re-exposed to antigen and start to produce helper or suppressor cytokines, or mediate cytotoxicity. Phenotypically, they share characteristics of antigen-activated cells; they are l-selectinlo, α4β7, CD25lo and some are DR-positive. They are non-dividing, virtually all express Fas and some are FasL+. (MacDonald 2003)

### 2.4.2.2 Lamina propria B cells

At birth, there are no LP plasma cells (Perkkio & Savilahti 1980) but the intestinally protective IgA molecules are received externally, via breast milk. IgA is the major immunoglobulin isotype in intestinal secretions (Crabbe & Heremans 1968). In human adult jejunum, around 80% of plasma cells secrete IgA (Crago et al. 1984).

### 2.4.3 Enterocytes and M-cells

The gut epithelium seems to be able to play an immunomodulatory role. The small bowel villous enterocytes express MHC class I and II molecules (Hershberg & Mayer 2000). In man, damaged enterocytes can rapidly up-regulate stress-induced MHC-like genes resulting in their lysis by γδ T cells (Groh et al. 1998). Recently it has been shown in mice that antigen expression by enterocytes is sufficient to trigger a specific CD4+ T cell response leading to mucosal infiltration (Westendorf et al. 2005). IL-7, a growth factor for LPL cells, is also secreted by enterocytes (Watanabe et al. 1995). IgA synthesised by LP plasma cells is transported to the mucosal surface by enterocytes.

PPs are partly covered by specialized microvillus epithelial cells - M cells. Under the transmission electron microscope, M cells are seen to be invaginated by enfolded lymphocytes and stretched in thin bands between adjacent enterocytes. The absorbed food antigens, which reach the luminal surface of M cells, are taken up by pinocytosis, carried in vesicles, and released into the intercellular space beneath the M cells, where they can be directly taken up by lymphocytes. Particles are also phagocytized by macrophages within or beneath the epithelium, which migrate into the lymphoid follicles of PPs, where antigens are presented to lymphocytes. The IgA-mediated transportion mentioned previously is absent in both the M cells and the enterocytes of follicle associated epithelium (Owen 1999).
In addition to the presence in duodenal LP of patients with non-IgE-mediated cow's milk hypersensitivity (Ulanova et al. 2000), the CD1d molecule is also expressed by gut epithelial cells (Blumberg et al. 1991). CD1d seems to be a ligand for IELs and intestinal CD8+ T cells (Balk et al. 1991, Panja et al. 1993).

### 2.4.4 Mucosal mediators

A substantial proportion of normal IELs and LPLs secrete interferon-gamma (IFN-γ) and/or interleukin-4 (IL-4). These cytokines are probably involved in the normal homeostasis of human intestinal mucosa. (Carol et al. 1998)

CD30 may identify Th2-type clones, but its relevance in vivo is still a matter of debate, as high serum levels of soluble CD30 have been found in both Th1- and Th2-dominated disorders (Horie & Watanabe 1998). Actiuated lymphocytes and NKs release CD30 (Del Prete et al. 1995). Increased levels have been detected in respiratory allergies (Blanco Quiros et al. 1999) and in infants with IgE-mediated CMA. Cow’s milk protein-specific T cells express much higher levels of CD30 than the same cells in healthy infants (Schade et al. 2002).

In CMSE, Hauer et al. detected an increase in both IFN-γ and IL-4-secreting LP cells; the cells secreting IFN-γ being 10 times more numerous than cells secreting IL-4, supporting a dominance of Th1-type responses. In serum samples, significantly increased IFN-γ and IL-4 levels were found by the ELISPOTs method. Compared to controls, IL-5 ELISPOTs were unchanged but IL-10 ELISPOTs were reduced in CMSE. In IgE-mediated CMA, IL-4 ELISPOTs were significantly greater compared with those with CMSE (Hauer et al. 1997). In addition to IFN-γ expression of duodenal LPLs, increased cryptal HLA-DR expression was observed in children with delayed-type of food allergy (milk and/or cereals), supporting the idea of mucosal Th1 dominance (Veres et al. 2003).

CD patients and cases with potential CD have shown to have higher LP densities of IL-2, IL-4 and IFN-gamma positive cells, suggesting that inflammatory markers can be identified long before villous changes are visible (Westerholm-Ormio et al. 2002). However, the major Th1 inducing cytokine, IL-12, is undetectable in these tissues and the mechanism by which Th1 effector cells are generated remains unknown. Interferon-alpha (IFN-α), a cytokine capable of promoting IFN-γ synthesis, has been implicated in the development of Th1 mediated immune diseases. There is a case report by Monteleone et al (2001) of a patient receiving IFN-α for chronic myeloid leukaemia, who developed CD-like enteropathy with villous atrophy, crypt cell hyperplasia, and a high number of CD3+ IELs. The serological antibodies were positive. RNA analysis revealed pronounced expression of IFN-γ. Gluten withdrawal resulted in a patchy improvement of mucosal morphology, normalisation of laboratory parameters, and resolution of clinical symptoms. With Western blot analysis, mucosal IFN-α expression has been seen in untreated CD patients but not in normal cases that have been shown to express IFN-γ. (Monteleone et al. 2001)
2.5 Cell-mediated cytotoxicity

As early as the 1950s, morphological distinctions between targets attacked by lymphocytes vs. targets attacked by antibody and complement were established (Kalfayan & Kidd 1953). Later it was demonstrated by isotope release assays that antibody and complement damage was restricted to the plasma membrane, whereas lymphocytes produce a more general internal disintegration of the target cell, including the nucleus (Russell et al. 1980).

NKs and CTLs, the two major populations of cytotoxic lymphocytes (CLs), are key components of the innate and adaptive cytotoxic immune responses. Both can destroy their target cells by either the PFN/granzyme or the Fas/FasL pathway; the two major known pathways of cell-mediated cytotoxicity inducing programmed cell death - apoptosis. A modern way to consider the two pathways is to separate those initiated by Fas-associated death domain (FADD) through a target cell receptor from those that require PFN. (Russell & Ley 2002) However, in vivo transplantation and graft vs. host disease (GvHD) experiments across MHC class I or II (CD8+ and CD4+ effectors, respectively) suggest that the PFN/granzyme pathway dominates in the class I elimination pathway and that Fas/FasL dominates in class II elimination (Schulz et al. 1995, Graubert et al. 1997).

2.5.1 Granule exocytosis pathway

Cytotoxic granules are specialized lysosomes. After protein synthesis, important posttranslational modifications occur. FasL appears to be stored in the same cytotoxic granules as perforin and granzymes, and may in fact be delivered to the target cell Fas receptor by the same granules that deliver other cytotoxic granule components (Bossi & Griffiths 1999). The granules are oriented towards the region of receptor activation (NK/TCR) and release the granule components into the space, which is initiated by the receptor but maintained by adhesion molecules. At the cell surface, cytotoxic granules fuse with the plasma membrane and the contents are secreted into the tight intracellular junction between the cells. (Atarashi et al. 2005) This “immunological synapse” forms a tight seal that directs granules to the target cell and keeps them from leaking out and causing damage. Where there is intense T cell activation, for example in rheumatoid arthritis, some of the molecules leak out in small concentrations (Spaeny-Dekking et al. 1998). If leakage occurs, several auto-protective mechanisms exist to protect CTLs and NKs from membrane damage (Russell & Ley 2002).

The PFN-dependent pathway is dominant in CD8+ CTLs and natural killer (NK) cells. NK cells are part of the innate immune system and exhibit a rapid response to challenge, but they generally do not proliferate significantly in response to antigen stimulation. Granules are preformed, although NK activity can be increased by cytokines like IL-2 and IFN-γ. In contrast, naive CTL precursors have no cytotoxic activity. After an antigen challenge, TCR-stimulated activation and proliferation processes are initiated that also require cytokine receptors. These processes require 1–3 days to reach maximal activity.
and include the induction of granule components such as PFN and granzymes. (Russell & Ley 2002)

2.5.1.1 Perforin

PFN is a protein found in the cytoplasmic granules of CTLs that are involved in cell-mediated immune responses inducing target cell death. Perforin is physiologically expressed in human CD3+, CD56+ NKs, CD3 positive large granular lymphocytes and γδ-TCR bearing T cells. In CD8 positive T cells, PFN expression is significantly induced after a challenge. (Lichtenheld et al. 1988)

PFN is synthesized as a 70-kDa precursor that is cleaved at the carboxyl terminus to yield the active 60-kDa form (Uellner et al. 1997). In the presence of calcium, perforin polymerizes and probably enters the target cell membrane via phosphocholine, a specific calcium-dependent receptor molecule (Tschopp et al. 1989). It has been suggested that Nks are able to release lysolipid platelet activating factor that has a synergistic action with PFN, producing membrane damage (Berthou et al. 2000).

Several experiments suggest that perforin alone is incapable of causing target cell apoptosis unless molecules like the granzymes are also present. Previously, PFN was thought to form a channel through which the other apoptotic granule proteins pass into the target cell cytoplasm, but the size of this pore is probably too small to permit large molecules like granzymes to enter target cells. (Browne et al. 1999)

2.5.1.2 Granzyme A and B

Granzymes range in size from approximately 30 to 65 kDa, they are probably complexed with serglycin upon secretion, which may serve to protect them from inactivation (Spaeny-Dekking et al. 2000, Metkar et al. 2002). In addition to causing damage to the target cell cytosol, granzymes rapidly accumulate in the nucleus, where they can activate nuclear damage pathways (Jans et al. 1998, Trapani et al. 1996) independently or synergistically in a perforin-dependent manner (Nakajima et al. 1995). There is also evidence of synergy between GrA and GrB (Nakajima et al. 1995). A few hours after entry of granzymes, target cells show morphological features of apoptosis with chromatin condensation and nuclear fragmentation (Beresford et al. 1999, Zhang et al. 2001).

Granzyme A (GrA) was the first serine proteinase discovered in cytotoxic granules (Pasternack & Eisen 1985). It turned out to be the most abundant protease in NKs and CTLs (Lieberman 2003). In CTLs, in which granule expression is strongly induced by activation (unlike NK cells that have constitutive expression), GrA expression continues long after stimulus removal (Garcia-Sanz et al. 1990). GrA induced deoxyribonucleic acid (DNA) damage results in large DNA fragments that are not detected by the usual apoptosis assays (Lieberman & Fan 2003). When GrA is delivered into target cells with PFN, apoptosis occurs in minutes (Beresford et al. 1999). An important target of GrA is a newly discovered complex, the SET complex, which is thought to be important in the repair response to oxidative stress. By cutting three of the proteins in this SET complex,
GrA stimulates a deoxyribonuclease (DNAase) in the complex to make single-stranded DNA cuts and disable the target cell’s ability to repair the damage. In addition, GrA dissolves the nuclear lamina and targets histones. GrA also disrupts mitochondrial function and the integrity of the plasma membrane by as yet unknown mechanisms. (Lieberman & Fan 2003)

Extracellularly, GrA seems to enhance inflammation by cleaving the propeptide from a proinflammatory cytokine IL-1β, (Irmler et al. 1995), activating macrophages (Sower et al. 1996a) and inducing IL-8 production in intestinal epithelial cells (Sower et al. 1996b), the latter phenomenon is seen in inflammatory bowel diseases (Daig et al. 1996).

Recent evidence demonstrates that cells exposed to either monomeric granzyme B (GrB) or GrB-serglycin complexes readily internalize the granzyme and undergo apoptosis in the absence of Mrp300 and Mrp46 (Dressel et al. 2004). In the target cell, free GrB initiates cell death by cleaving a variety of protein substrates that are either directly or indirectly linked to the induction of DNA fragmentation and apoptosis (Zhang et al. 2001, Lieberman 2003).

2.5.1.3 TIA-1

T cell-restricted intracellular antigen type 1 (TIA-1) and its closely related homologue TIAR are members of the RNA recognition motif (RRM) family of RNA binding proteins (Kawakami et al. 1992, Anderson et al. 2004). In peripheral blood, TIA-1 is expressed in about 50% of CD8+ T cells and 5% of CD4+ T cells but is also found in CD4+ and CD8+ activated T cell clones, and in activated NK cell clones (Anderson et al. 1990). With immuno-electron microscopy, TIA-1 has been localized to the membranes of cytotoxic granules (Medley et al. 1996). TIAR seems to be concentrated in the nucleus but then moves from the nucleus to the cytoplasm within 30 min after Fas ligation, which is thought to be a feature of the apoptotic program (Taupin et al. 1995). TIA-1 and TIAR function as translational silencers that seem to influence the duration of stress induced translational arrest (Kedersha et al. 1999, Piecyk et al. 2000). As discussed by Anderson (2000), by controlling the duration of translational arrest, TIA-1 and TIAR might determine whether stressed cells live to repair the stress induced damage or die by apoptosis (Anderson et al. 2004). Previous results show that TIA-1 and TIAR can independently and selectively regulate the production of TNFα (Piecyk et al. 2000) in macrophages but not in activated lymphocytes (Saito et al. 2001).

In patients with active CD, the density of TIA-1 positive cells is increased (Russell et al. 1993, Drut & Drut 2004) and the localization of TIA-1 has been restricted to lymphocytes by immunofluorescence (Zimmer et al. 1998).

2.5.2 Fas/FasL system

Fas (APO-1 or CD95) is a cell surface glycoprotein expressed on rapidly proliferating cells. The Fas/FasL pathway appears to be active mostly in CD4+ cells, especially those of the Th1 phenotype (Ju et al. 1995) but also in innate NKs (Smyth et al. 2005). An
The important difference between the FADD and PFN-initiated cytotoxic pathways is the increased speed of the apoptotic event in the latter. Once formed, granules can be reoriented and released within minutes of TCR stimulation (Kupfer et al. 1985). By contrast, very little FasL is stored, even in activated cells. Therefore, maximal activity requires the induction of a new ligand over a 1–2 h period after TCR stimulation.

Activation of the Fas receptor leads to recruitment and activation of caspase 8, which activates caspase 3, leading to a cascade of downstream events (DNA cleavage, cell membrane changes, etc), finally causing apoptosis. Caspase 3 can also be activated by a complex of proteins, including caspase 9, apoptotic protease-activating factor-1 (APAF-1) and cytochrome C. This pathway is activated by leakage of cytochrome C from the mitochondria, which is regulated by the Bcl-2 family of proteins (Peppelenbosch & van Deventer 2004).

2.5.2.1 Activation induced cell death of T cells

In addition to target cell apoptosis induced by the Fas/FasL pathway, activated T cells also enter apoptosis, a process known as activation-induced cell death (AICD). Intrinsic defects in the control of apoptosis in mucosal T cells are strongly implicated in the pathogenesis of inflammatory bowel disorders (Peppelenbosch & van Deventer 2004).

Initially, T cells are resistant to apoptosis, but after several days they develop sensitivity due to repeated TCR-stimulation (Suda et al. 1996). The ligation of Fas onto activated T cells by either Fas antibodies or recombinant human FasL also results in apoptosis (Ju et al. 1995). A causal relationship between these two mechanisms has been reported. The stimulation of previously activated T cells was reported to result in the expression of FasL mRNA (Alderson et al. 1995) and FasL antagonists were observed to be able to inhibit AICD (Brunner et al. 1995), indicating that AICD of previously stimulated T cells is mediated by Fas/FasL interactions. The change in propensity to undergo AICD has been attributed to an initially high level of Fas associated death domain protein–like interleukin 1beta converting enzyme –like inhibitory protein (FLIP) expression that diminishes over time (Irmler et al. 1997). IL-2, which is produced by activated T cells and considered important for their survival and proliferation (Vella et al. 1998), has been reported to be required for the reduction in FLIP expression (Refaeli et al. 1998). Therefore, reduction in cytokine levels can result in AICD (Strasser et al. 1995, Marsden & Strasser 2003).
3 Aims of the study

Atopic diseases are becoming increasingly common worldwide, and food hypersensitivities seem to be a part of this rise. Previous studies indicate that cell mediated immunity has a role in delayed paediatric gastrointestinal food hypersensitivities, but the exact pathogenetic mechanisms are unknown. CMSE is a non-IgE related type of food hypersensitivity with variable gastrointestinal symptoms and with no mucosal damage. Open or blinded elimination/challenge tests, are currently the mainstay of diagnosis, endoscopic, histological and laboratory findings being largely non-specific.

Therefore, we investigated the mucosal and serum changes related to a particular type of cell mediated immunological activation, T and NK cell cytotoxicity, to see whether this activation has an important role in the pathogenesis of CMSE. We also wanted to see whether these analyses could be used in the diagnostics of CMSE, with a view to developing a non-invasive diagnostic test. For comparison, we studied CD, where cytotoxic activation of T cells is known to play an important role in the pathogenesis.

The specific aims of the study were:

1. To assess the expression of cytotoxic TIA-1 in the mucosal epithelium and LP of the gastric antrum and descending duodenum in children with untreated or treated CMSE.
2. To assess the expression of PFN, GrA and GrB in the mucosal epithelium of the duodenal bulb and descending part of the duodenum in children with CMSE and CD.
3. To determine the rates of apoptosis and proliferation in the mucosa of the duodenal bulb and in the descending part of the duodenum in children with CMSE and CD.
4. To look for evidence of increased mucosal cytotoxic activation in children with CMSE and CD that can be detected non-invasively by the analysis of serum concentrations of GrA, GrB, CD30 and sFas.
4 Material

4.1 Patients

158 preschool and school aged children were sent for investigation of refractory gastrointestinal complaints to Oulu University Hospital between the years 1995-2000. All subjects suspected to have active food hypersensitivity on the basis of prolonged diarrhoea and/or abdominal pain were scheduled for oral challenges with milk, wheat or oats, each continuing for two weeks if symptoms did not manifest earlier. CMSE was diagnosed either with a blind or open elimination-challenge test, that resulted in the disappearance of symptoms on a milk elimination diet (2 weeks) and their reappearance in an open challenge test with low lactose milk products. The elimination test continued for another two weeks if symptoms did not manifest earlier. Abdominal pain, diarrhoea or loose mucous stools, and exacerbation of dermatitis were each considered as a positive result. In cases with a history of atopy such as treatment resistant dermatitis, asthma or allergic rhinitis, the possibility of IgE mediated milk allergy was screened for by determination of milk-specific IgE concentration (RAST-test).

The same set of patients was used in the immunohistochemical studies analysing the rate of apoptosis (III) and expression of cytotoxic granules (II). In the serum study (IV), 22 patients had endoscopy either before or after an elimination diet and the small intestinal samples of these cases were included in the immunohistochemical studies (II, III), as well.

4.1.1 Patients with cow’s milk protein sensitive enteropathy

57 patients with various symptoms (most commonly abdominal pain,) were not on a milk elimination diet at the time the biopsy or serum samples were taken. The diagnosis of these untreated CMSE cases was confirmed later by the milk elimination and challenge test. If a patient had had occasional blood in stools, a colonoscopy was scheduled as well.


Unspecific LNH of the terminal ileum was the only macroscopic finding in the studied children but only patients with no definite large intestinal disease were included.

4.1.2 Patients with treated cow’s milk protein sensitive enteropathy

The group of treated CMSE subjects consisted of patients that were on an elimination diet at the time of endoscopy, but continued to have some persistent gastrointestinal symptoms such as prolonged diarrhoea or abdominal pain.

4.1.3 Patients with celiac disease

Altogether 24 children with CD were studied and treated as the disease control group. CD was diagnosed according to the acknowledged criteria (Walker-Smith et al.) and only those with subtotal or total villous atrophy with crypt hyperplasia on duodenal biopsy were included. Referral symptoms were diarrhoea or loose mucous stools, and abdominal pain.

4.1.4 Control cases

Altogether there were 52 control cases in the immunohistochemical and serological studies. Forty cases were examined thoroughly for various gastrointestinal symptoms including the milk elimination and challenge test but were not found to have any significant gastrointestinal disease. 12 children taken part in the first paper studying TIA-1 expression (I) were referred for a consultation for feeding or swallowing difficulties and also in these control cases, long term follow-up had excluded any somatic gastrointestinal disease and the symptoms were thought to be of psychological origin. The non-gastrointestinal symptoms of these cases did not indicate a need for an elimination and challenge test and the symptoms subsided during follow-up of at least six months duration on a milk containing diet.

Table 2. Patients

<table>
<thead>
<tr>
<th>Original paper</th>
<th>CMSE untreated (mean age)</th>
<th>CMSE treated (mean age)</th>
<th>CD (mean age)</th>
<th>CTRL (mean age)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>27 (7.8)</td>
<td>15 (6.9)</td>
<td>-</td>
<td>24 (8.3)</td>
</tr>
<tr>
<td>II, III</td>
<td>21 (9.7)</td>
<td>15 (11.2)</td>
<td>18 (10.7)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>18/4* (9.3)</td>
<td>5/3 (9.0)</td>
<td>20/11 (10.5)</td>
<td>14/4* (11.4)</td>
</tr>
<tr>
<td>total</td>
<td>57</td>
<td>18</td>
<td>24</td>
<td>52</td>
</tr>
</tbody>
</table>

CMSE (cow’s milk protein sensitive enteropathy), CD (celiac disease), CTRL (control) *number of cases from the studies II and III
4.2 Tissue specimens

Upper intestinal endoscopy was performed with an Olympus GIF-IT140 endoscope under general anaesthesia. Biopsy samples were taken for routine histology from the antrum of the stomach, the anterior wall of the bulb of the duodenum and from the descending duodenum below the sphincter of Oddi; each at the most active site when local pathology was seen.

4.3 Serum samples

Serum samples were collected between 1999 and 2000. The samples of untreated CMSE cases were taken while the diagnosis was being made unlike the samples of treated subjects, who were already on an elimination diet.
5 Methods

5.1 Traditional histology

Haematoxylin & eosin stained sections were re-evaluated by a pathologist (TJK) blinded to the clinical or endoscopic data. The biopsies were evaluated using established criteria (Perera et al. 1975). The gastric biopsies were also stained with modified Giemsa for the detection of *Helicobacter pylori* (I).

Villi and crypt heights were measured with an ocular micrometer. The concentration of eosinophilic leukocytes was counted separately in the surface epithelium, in the villous lamina propria of the duodenum, and the supra-glandular lamina propria in antral mucosa, and in the deep lamina propria. The number of eosinophilic leukocytes in both layers of the mucosa was counted in at least three random fields using a 40x objective lens (0.2 mm$^2$) and a mean for each was calculated. When tissue filled only a part of the measuring field, the number of cells was related to the actual area of mucosa in the field. The counts were expressed as eosinophils/ mm$^2$ tissue. The density of duodenal mononuclear inflammatory cells was semiquantitatively graded as follows: Grade 0: normal, scanty infiltrate; grade 1: slight increase; grade 2: moderate increase; grade 3: heavy increase.

5.2 Immunohistochemistry

Biopsies were fixed in 10% neutral buffered formalin and processed in paraffin (I-III). For all immunohistochemical stainings, the paraffin embedded specimens were sectioned at 4-5 µm and dried either overnight at 37°C or 60 minutes in 60°C. Pre-treatment of samples depended on the antibody used (Table 3). For control stains, primary antibody was replaced with phosphate buffered saline or antibody diluent (Dako, Glostrup, Denmark). The sections were counterstained with methyl-green or haematoxylin-eosin.

For frozen sections (IV), the biopsy samples from the bulb of the duodenum were embedded in an optimal cutting temperature compound (Miles Laboratories, Elkhart, IN,
USA), frozen in liquid nitrogen and stored at –70°C until analysis. Sections were cut to 5 µm and were fixed in methanol for 3 min at +4°C.

Table 3. The primary antibodies used in immunohistochemistry

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Manufacturer</th>
<th>Pre-treatment</th>
<th>Dilution and incubation</th>
<th>Protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIA-1, monoclonal (I)</td>
<td>Coulter, Corporation, Miami, Florida, USA</td>
<td>none</td>
<td>1:400, 60min, RT</td>
<td>streptABComplex (Dako)</td>
</tr>
<tr>
<td>TIA-1, monoclonal (II)</td>
<td>Coulter, Corporation, Miami, Florida, USA</td>
<td>none</td>
<td>1:800, 60min, RT</td>
<td>Ultravision Large Volume Detection System, LabVision autostainer™, Fremont, CA</td>
</tr>
<tr>
<td>CD3, polyclonal (II)</td>
<td>Code A 0452; Dako, Glostrup, Denmark</td>
<td>10 ml/L citrate buffer, 20 min, RT</td>
<td>1:500, 60min, RT</td>
<td>EnVision™, Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>TIA-1, monoclonal (II)</td>
<td>Coulter, Corporation, Miami, Florida, USA</td>
<td>none</td>
<td>1:800, 60min, RT</td>
<td>Ultravision Large Volume Detection System, LabVision autostainer™, Fremont, CA</td>
</tr>
<tr>
<td>CD3, polyclonal (II)</td>
<td>Code A 0452; Dako, Glostrup, Denmark</td>
<td>10 ml/L citrate buffer, 20 min, RT</td>
<td>1:500, 60min, RT</td>
<td>EnVision™, Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>PFN (II)</td>
<td>Clone 5B10; Novocastra, UK</td>
<td>Tris-EDTA (pH 9) with 850W for 2min and with 300W for 15min.</td>
<td>1:20, 60 min, RT</td>
<td>EnVision™, Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>GrA (II)</td>
<td>CLB, Clone CLB-GA6.</td>
<td>trypsin (pH 7.8), 10min, 37 degrees</td>
<td>1:200, 60 min, RT</td>
<td>EnVision™, Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>GrB (II)</td>
<td>CLB, Clone CLB-GB7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ki-67 (III)</td>
<td>clone MIB-1, PharMingen, San Diego, CA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TUNEL (III)</td>
<td>Integren Company®, Oxford, UK</td>
<td>20 µg/ml proteinase K (Sigma, Dorset, UK), 15 min, RT</td>
<td>1:50, 60 min, RT</td>
<td>ApopTag™</td>
</tr>
<tr>
<td>M30 CytoDEATH (III)</td>
<td>Clone M30; Roche Diagnostic Division, Helsinki Finland</td>
<td>Tris-EDTA (pH 9), with 850W for 2min and 300W for 15min.</td>
<td>1:900, 60 min, RT</td>
<td>EnVision™, Dako, Copenhagen, Denmark</td>
</tr>
<tr>
<td>αβ-TCR (IV)</td>
<td>MAb βF1; Endogen Inc., MA, USA</td>
<td>normal horse serum, 1:20, 20min</td>
<td>1:40</td>
<td>Vectastain Elite ABC kit PK 6102, Vector Laboratories, Burlington, MA, USA</td>
</tr>
<tr>
<td>γδ-TCR (IV)</td>
<td>Mab TCR-γ; Endogen Inc.</td>
<td></td>
<td>1:140</td>
<td></td>
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<tr>
<td>CD3 (IV)</td>
<td>mAb Leu-4; Becton Dickinson, San Jose, CA, USA</td>
<td></td>
<td>1:15</td>
<td></td>
</tr>
</tbody>
</table>

5.2.1 Counting of positive cells

Immunohistochemical cell counting was performed by the author, whilst blinded to clinical information and patient group. Except for the first paper (I), the stained specimens were evaluated with a light microscope using 100x magnification and immersion oil. Cell counting was performed in every villus of specimen that had been sectioned through its entire length, from tip to base. Villi overlying lymphoid follicles
were not counted, as they were usually deformed to some degree. The indices were calculated as a ratio between positive cells and epithelial cells and indicated as counts/100 epithelial cells. To obtain information on relative numbers of cells expressing cytotoxic granules in different parts of the villous epithelium, we divided the villus into thirds (upper, middle and lower), based on the number of epithelial cells (Figure 1). We calculated the ratio of positive cells and all epithelial cells in each third using a spreadsheet program (Excel). When severe villus atrophy was seen, as was the case in most CD subjects, but none of CMSE or control cases; only the overall, not the one-third indices were counted.

In the paper concerning apoptosis (III), the number of positive cells in villous LP was counted as well. The approximate area of the whole villous LP was calculated from the measured height and width of each villus, and the density of positive cells in the whole LP and separately in upper, middle and lower third was expressed as cells/mm². Again, where there was severe villous atrophy, only the overall, not the one-third indices were counted.

Ki-67 positive and negative epithelial cells were counted only on optimally cut half-crypt units, and the proportion of positive cells (%) was used for comparison of proliferation activity (III).

**5.3 Studies on serum samples**

Serum samples were evaluated by the ELISA method for GrA and GrB (Pelikine human granzyme A, and Pelikine human granzyme B; CLB, Amsterdam, Netherlands), serum soluble Fas (R&D Quantikine human sFas; Minneapolis, Minnesota, USA), and CD30 (Bender MedSystems GmbH, Vienna, Austria). All samples were analysed simultaneously using the manufacturer’s instructions. GrA and GrB concentrations were expressed as units/ml, and those of sFas and CD30 as pg/ml.

**5.4 Statistics**

The data were analyzed with the SPSS 10.1 package (SPSS Inc., Chigaco, USA). Since the continuous variables showed a skewed distribution, the nonparametric Kruskal-Wallis test was used to compare indices between the groups. Within-case differences between the different parts of the villi, the duodenal bulb and the descending duodenum were compared by the Wilcoxon signed rank test. The relationships between cell counts were analysed by Spearman’s 2-tailed rank correlation test; the Kruskall-Wallis and Fisher’s exact tests being used for dichotomous variables. All statistical tests were two-sided, and a p-value of 0.05 or less was considered significant. The Bonferroni correction for multiple tests was not used to avoid an over-conservative analysis (Perneger 1998).
5.5 Ethical considerations

Following a verbal explanation of the study plan, written parental consent was obtained for all children. The protocol was approved by the Ethical Committee for Clinical Science of Oulu University Hospital.
6 Results

6.1 Endoscopic and histological findings (I, II, III, IV)

LNH of the duodenal bulb was seen more often among CMSE patients than in control subjects (p=0.000). Compared to CD, endoscopic esophagitis was more common among CMSE patients (p=0.014). The main macroscopic findings of untreated CMSE patients, subjects with CD and controls are summarized in Table 1.

There were no significant routine histological differences between CMSE cases and controls. This included eosinophil counts and the estimated density of LP mononuclear cells either in the duodenal bulb or in the descending duodenum. None of the CMSE patients or controls had villous atrophy or crypt hyperplasia which is typically seen in CD cases. The gastric biopsies were negative for Helicobacter pylori.

Table 4. Endoscopic findings

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Esophagitis</th>
<th>Gastritis</th>
<th>LnH; bulbus</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMSE</td>
<td>71</td>
<td>11(5%) P1</td>
<td>15(21%)</td>
<td>33(46%) P2</td>
</tr>
<tr>
<td>CD</td>
<td>35</td>
<td>0</td>
<td>3(9%)</td>
<td>2(6%)</td>
</tr>
<tr>
<td>CONTR</td>
<td>44</td>
<td>4(9%)</td>
<td>6(14%)</td>
<td>2(5%)</td>
</tr>
</tbody>
</table>

P1=0.014 compared to CD; P2=0.001 compared to controls; Chi-square Test

6.2 Immunohistochemistry

6.2.1 CD3+ IELs and TCRs (II, III, IV)

The densities of CD3+ cells were significantly higher in CMSE patients than in control subjects in the villus epithelium of both the bulb and the descending duodenum. In the descending duodenum, analysis of villi in thirds (Figure 2 in Methods section) showed
that in addition to the accumulation of CD3+ IELs in the villous tip region, CD3+ expression was also a characteristic of the lower epithelium (Table 5). The CD patients showed a significant, heavy increase of CD3+ IELs in both regions (II; Table 1).

On frozen sections, the untreated CMSE patients had significantly higher densities of αβ- and γδ- TCR positive T cells in the duodenal bulb compared to the controls. However, in all five CMSE cases on a diet, the densities were comparable with the controls (IV; Table 1).

### 6.2.2 Cytotoxicity related markers (I, II)

The cytotoxic markers TIA-1, GrA, GrB, and PFN showed a cytoplasmic, granular staining. The morphology of positive cells was lymphoid. In CD, the number and intensity of cytotoxic granules in each positive cell seemed somewhat increased in comparison with the other groups, but this was not analysed systematically.

#### 6.2.2.1 TIA-1

In untreated CMSE subjects, the number of epithelial TIA-1 expressing cells in the descending duodenum was significantly increased compared with treated patients and controls (I; Table 3 and II; Table 2). On the other hand, the density of TIA1 positive cells in the subjects on an elimination diet did not differ significantly from the controls (I; Table 3). TIA-1 expression in the duodenal bulb was a lot higher in CD, compared to CMSE. There no differences between CMSE patients and controls in this area (II; Table 2). Untreated patients with CMSE tended to have more TIA-1+ lymphocytes in gastric LP, but this finding was only found to be significant in the superficial antral mucosa when compared with the controls having rapid abdominal pains (I; Table 3). In all groups, there was a strong correlation between the counts of TIA-1 positive IELs and LPLs in antral and duodenal mucosa (C= 0.613-0.658; p>0.001) (I)

#### 6.2.2.2 Perforin

The number of PFN+ IELs in descending duodenum was significantly higher in the CMSE group than in controls. Also, the villous tip epithelium of the bulbus showed increased numbers of positive cells compared to controls. In CD, the increment was seen compared both to controls and CMSE. (II; Table 3)

#### 6.2.2.3 Granzyme A

In CMSE, GrA expression in the descending duodenum was increased in the villous tips and basal thirds, but not in the middle thirds (Table 5). In CD, GrA+ containing cells were seen in the bulbus and in the descending duodenum (II; Table 4). Unlike with TIA-1 and
PFN, comparison of CMSE and CD showed that in addition to increased numbers of positive cells, the GrA^+\text{/}CD3^+ ratio in the descending duodenum was higher in CD than in CMSE (II; Table 6). In the bulb the ratio was similar.

6.2.2.4 Granzyme B

Neither the GrB^+ density nor the ratio of GrB^+\text{/}CD3^+ cells was significantly increased in CMSE. In CD however, GrB expression was seen in both regions and the difference was significant compared both to controls and CMSE (II; Table 5). In the descending duodenum, the GrB^+\text{/}CD3^+ proportion in CD subjects was higher than in CMSE patients (II; Table 6) and with GrB the same phenomenon was found in the bulb.

6.2.2.5 Regional variation and distribution of cytotoxicity related markers (I, II)

In CMSE, in addition to the overall villous epithelium indices, the upper, middle and lower third compartments of descending duodenal villi had independently increased levels of CD3, TIA-1, PFN, and GrA positive granules compared to controls (Table 5). A graphical representation of IEL distribution in the descending duodenum shows trends between the different markers (Figure 1).

Some variation in the regional distribution of cytotoxic marker densities was seen in CMSE: the densities of TIA-1 and GrA expressing cells were significantly higher in the descending duodenum than in the bulb (I; Table 3 and II; Table 2, 4). PFN showed a trend towards a similar pattern (II; Table 3). In the antrum, the epithelial TIA-1 density was not increased (I; Table3). In CD and in the controls, no significant differences between the bulb and descending part were seen with any of the markers.
Table 5. The ratios between CMSE and control cases based on the indices of positive IELs of descending duodenum per 100 epithelial cells. Median (min-max).

<table>
<thead>
<tr>
<th>Marker</th>
<th>Area</th>
<th>Ratio</th>
<th>CMSE N=21</th>
<th>Controls N=18</th>
<th>CD N=15</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD3</td>
<td>Whole villus</td>
<td>1.6</td>
<td>32.0 (13.4-55.1)</td>
<td>20.4 (11.1-38.7)</td>
<td>68.5 (14.6-93.6)</td>
</tr>
<tr>
<td></td>
<td>Upper third</td>
<td>1.3</td>
<td>30.4 (17.7-57.1)</td>
<td>22.6 (15.8-42.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower third</td>
<td>1.4</td>
<td>23.6 (7.1-58.2)</td>
<td>16.4 (4.2-35.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIA-1</td>
<td>Whole villus</td>
<td>2.4</td>
<td>12.3 (1.9-26.3)</td>
<td>5.2 (0.4-17.9)</td>
<td>26.6 (2.6-63.7)</td>
</tr>
<tr>
<td></td>
<td>Upper third</td>
<td>2.1</td>
<td>15.6 (2.5-32.1)</td>
<td>7.4 (1.2-29.2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td>2.5</td>
<td>12.5 (0-27.6)</td>
<td>5.1 (0-16.9)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower third</td>
<td>2.8</td>
<td>10.5 (1.9-26.9)</td>
<td>3.7 (0-10.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFN</td>
<td>Whole villus</td>
<td>1.7</td>
<td>16.3 (4.6-42.6)</td>
<td>9.5 (3.5-25.2)</td>
<td>40.3 (10.2-76.9)</td>
</tr>
<tr>
<td></td>
<td>Upper third</td>
<td>1.6</td>
<td>17.3 (9.9-41.5)</td>
<td>11.1 (0-25.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td>1.8</td>
<td>20.4 (3.6-45.0)</td>
<td>11.3 (1.2-35.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower third</td>
<td>2.0</td>
<td>14.3 (0.9-41.9)</td>
<td>7.1 (1.7-16.7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GrA</td>
<td>Whole villus</td>
<td>1.4</td>
<td>19.6 (2.6-44.7)</td>
<td>14.1 (4.7-33.3)</td>
<td>63.6 (9.9-121.4)</td>
</tr>
<tr>
<td></td>
<td>Upper third</td>
<td>1.4</td>
<td>25.7 (1.5-63.8)</td>
<td>18.2 (4.7-34.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Middle third</td>
<td>1.4</td>
<td>20.6 (0-42.8)</td>
<td>15.1 (1.3-62.1)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lower third</td>
<td>2.0</td>
<td>19.0 (1.9-32.6)</td>
<td>9.4 (1.9-16.7)</td>
<td></td>
</tr>
</tbody>
</table>

* p-value < 0.05 compared to controls

Fig. 2. A graphical demonstration of upper (U), middle (M) and lower (L) villous indices (positive cells per 100 epithelial cells) in the descending duodenum.
6.2.3 Markers related to proliferation and apoptosis

TUNEL positivity was seen either as complete nuclear staining or as small TUNEL positive apoptotic bodies (III, Figures E, I). M30 demonstrated an extensive cytoplasmic reaction. In some cases macrophage-like cells of the LP showed positive labelling for M30 and TUNEL in their cytoplasm.

6.2.3.1 Ki-67

In CD patients, the cell proliferation in the crypt epithelium of the descending duodenum was significantly increased. In CMSE patients there was no significant difference in the rate of proliferation as compared with the controls (III; Table 5). Neither did we find any association with the apoptosis rate, based on TUNEL or M30 staining.

6.2.3.2 TUNEL

In the case of the TUNEL+ IELs it was difficult to see whether the cells were epithelial or lymphoid in origin; the positive cells in the LP cells were always of lymphoid morphology.

In the CMSE subjects, there were significantly more TUNEL+ cells in the villous epithelium of the descending duodenum compared with the controls, the difference being most evident in the middle third of the villous epithelium. In CD patients, the TUNEL indices of the descending duodenum were increased compared to both the controls and the CMSE patients (III; Table 2). Analysing the material as a whole, the epithelial TUNEL index correlated significantly with the overall LP counts in both the bulb and descending duodenum. The correlation was most evident in the tips of the villi of both areas. In the subgroup analyses, the epithelial and the LP TUNEL+ counts did not show any significant correlation (III).

6.2.3.3 M30

Consistent with the idea that TUNEL labels all apoptotic cells whilst M30 labels only apoptotic epithelial cells, the number of M30+ cells was always less than that of TUNEL+ cells in both the bulb and the descending duodenum, the difference between the counts being largest in CD and least in the control group, although the differences were minimal. (III; Table 4)
6.2.3.4 Regional distribution of apoptosis related markers between bulbus and descending duodenum

In CMSE subjects, TUNEL+ apoptosis was observed to be increased in the descending duodenum but in the bulbus, no significant difference from the controls was seen. Similarly in CD, the bulbus showed no increase (III; Table 2). Instead, a significantly increased index of M30+ positive epithelial cells was found only in the bulbus of CD patients (III; Table 4).

6.2.4 Correlation between immunohistochemical apoptosis and cytotoxicity related markers (II, III)

With CMSE patients’ samples from the descending duodenum, a positive correlation between epithelial M30 and GrB was seen (Table 2). In the villous tip region, GrA was associated with epithelial (M30) apoptosis (II; r=0.577, p=0.031). In the middle zone of the villous epithelium, where TUNEL+ apoptosis was most obvious in CMSE, a clear correlation between TUNEL and PFN was seen (II; r=0.527, p=0.036). In CD, the expression of TIA-1 was related to epithelial (M30) (r=0.483; p=0.002) and overall (TUNEL) apoptosis (II; r=0.495, p=0.001) and a similar trend was seen in CMSE with M30 as well (Table 5, descending duodenum and overall epithelial indices). In controls, epithelial apoptosis (M30) showed a positive correlation with GrA (II; r=0.595, p=0.019). In the bulb, overall TUNEL apoptosis was negatively correlated with perforin (I; r=-0.670, p=0.024).

Considering the whole group together, CD3 counts correlated significantly with both TUNEL+ and M30+ counts in the descending duodenum in both overall and upper third indices of epithelium (III). With the overall epithelial indices of descending duodenum, M30 had a strong positive correlation with TIA1 (r=0.483; p=0.002), GrA (r=0.513; p=0.001) and GrB (r=0.523; p=0.000). TUNEL associated similarly with TIA1 (r=0.495; p=0.001), GrA (r=0.57; p=0.000) and GrB (r=0.38; p=0.013) but also with PFN (r=0.57; p=0.000). In the bulbus, only TUNEL-TIA1 association was seen (r=0.376; p=0.048).

Table 6. The Spearman correlation table of CMSE children.

<table>
<thead>
<tr>
<th>CMSE</th>
<th>TIA-1</th>
<th>PFN</th>
<th>GrA</th>
<th>GrB</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>r=0.345; p=0.227</td>
<td>r=0.565; p=0.023</td>
<td>r=0.516; p=0.071</td>
<td>r=0.598; p=0.011</td>
</tr>
<tr>
<td>M30</td>
<td>r=0.445; p=0.074</td>
<td>r=0.046; p=0.864</td>
<td>r=0.428; p=0.127</td>
<td>r=0.531; p=0.028</td>
</tr>
<tr>
<td>TUNEL</td>
<td>r=-0.238; p=0.394</td>
<td>r=0.309; p=0.244</td>
<td>r=0.022; p=0.943</td>
<td>r=0.217; p=0.403</td>
</tr>
<tr>
<td>TIA1</td>
<td>r=-0.118; p=0.676</td>
<td>r=0.609; p=0.021</td>
<td>r=0.318; p=0.231</td>
<td>r=0.681; p=0.010</td>
</tr>
<tr>
<td>PFN</td>
<td></td>
<td></td>
<td>r=0.370; p=0.144</td>
<td></td>
</tr>
<tr>
<td>GrA</td>
<td></td>
<td></td>
<td></td>
<td>r=0.507; p=0.054</td>
</tr>
</tbody>
</table>
6.3 Serological studies

6.3.1 Serum concentrations of CD30, sFAS, Granzyme A and B (IV)

Subjects with untreated CMSE showed significantly increased concentrations of CD30 compared with controls and the treated CMSE group (IV; Figure 3). Serum sFas concentrations were similar to the control group, in untreated CMSE, treated CMSE and CD. Subjects with untreated CMSE showed significantly increased concentrations of GrA and GrB compared to controls and the treated CMSE group. In CD, these levels were similarly increased as compared to controls, but they remained somewhat lower than in the CMSE patients. (Figure 2) Four cases with CD showing patchy villous atrophy had significantly less GrA than CD patients with continuous villous atrophy. All the IEL subtype densities in these four patients were, however, similar to the remaining CD patients. (IV: Figures 1-3).

There was a strong positive correlation between serum GrA and GrB concentrations ($r=0.76$, $p<0.001$, Spearman) and between the granzyme concentrations and CD30 concentrations ($r=0.47$; $p<0.001$) in the whole study group. In sub-group analysis, the markers showed similar interdependence in CD ($p<0.026$). In CMSE, however, only GrA and GrB showed a significant correlation ($p<0.001$), and in the control group none of the markers showed any significant correlation.

![Graph showing proportions of subjects with serum GrA and GrB concentrations higher than the reference values for CTRL, CMSE, and CD](image)

Fig. 3. Proportion (%) of subjects with serum GrA and GrB concentrations higher than the reference values. None of the controls showed increased concentrations. The difference between controls and CD (GrA, $p=0.011$; GrB $p=0.032$) and controls and CMSE (GrA, $p=0.002$; GrB ($p=0.001$) were significant.
6.4 Correlations between immunohistochemical and serological findings

When all the subjects (N=57) are considered together, serum GrA shows a significant correlation with the numbers of all cell categories including CD3, αβ- and γδ-TCR-bearing IELs, while serum GrB correlated with the numbers of CD3 and γδ-TCR-bearing IELs. CD30 concentration showed significant correlation with all cell types, correlation with the numbers of γδ-TCR-bearing IEL being highly significant. (IV, Table 2) In contrast, sFas showed no significant relationship with cell counts as a whole, but in sub-group analysis, sFas and CD3 counts correlated in CD (r=0.513, p=0.021). In untreated CMSE, the number of γδ-TCR-bearing IELs correlated with CD30 concentration (r=0.707, p=0.01). In the control group the numbers of γδ-TCR-bearing IELs correlated with GrB (r=0.719, p=0.004).
7 Discussion

Cow’s milk proteins cause a spectrum of hypersensitivities, each with a characteristic age distribution and clinical characteristics, out of these the IgE-mediated cow’s milk allergy (CMA) is probably the best known. Non-IgE-mediated, cow’s milk related enteropathies exist in two clinically different entities: In infants, the term cow’s milk protein intolerance (CMPI) has been mostly used describing the severe form of enteropathy with mucosal damage that is improved by omitting cow’s milk from the diet. In preschool and school aged children, the term cow’s milk protein sensitive enteropathy (CMSE) is recommended. Except for the above mentioned CMPI cases, (Kuitunen et al. 1975, Chung et al. 1999), no evidence of epithelial or villous abnormality at light microscopic level has been seen in older children’s CMSE.

The bulk of pediatric allergy literature assumes that CMA generally remits by the age of five, with down-regulation of IgE-mediated hypersensitivity towards cow’s milk. In recent years, our group has shown that school-age children may have gastrointestinal symptoms that subside with the elimination of milk and dairy products and reappear during a challenge. Exposure to milk antigen seems to need to be for more than five days and a higher dose of milk protein is required than is generally used in elimination and challenge tests. This condition, CMSE, presents with delayed gastrointestinal symptoms. The variability and patchiness of macroscopic findings such as LNH, in CMSE make the diagnosis difficult, and a variety of tests may be needed to confirm it. The pathogenic mechanisms are poorly understood and the diagnosis often delayed due to a lack of specific clinical, histo-pathological and serological findings.

In this study we have further characterized the pathological features and pathogenetic mechanisms in school aged children with CMSE. We found that an increased number of IELs expressing TIA-1, GrA and PFN is characteristic of CMSE, confirming the importance of perturbed cell mediated immunity and lymphocyte cytotoxicity in this condition. In addition, the measurement of serum levels of GrA and GrB and CD30 might provide a novel, non-invasive method of monitoring immune-mediated intestinal diseases in both initial diagnosis and in the assessment of treatment response. Compared to CD, where lymphocyte cytotoxicity is an important pathogenic mechanism, the local increase in CMSE is less intense, there is no increase in GrB, and has a different anatomical distribution, being most intense in the descending part of the duodenum. Higher serum
granzyme concentrations in CMSE are possibly associated with an anatomically more extensive intestinal immune response.

7.1 Patients

Subjects with CMSE were all patients send to a pediatric gastroenterologist complaining of abdominal symptoms. This obviously causes some selection bias; subjects are likely representing the more severe end of the CMSE spectrum. Since endoscopy is an invasive operation in which children need to be anaesthetised, it is considered unjustified to investigate children with only mild symptoms by these methods. Therefore, it may not be possible to gather a series of mild cases of CMSE for a biopsy study. Due to similar ethical problems, the control series consisted of subjects with symptoms or conditions severe enough to warrant endoscopic investigation. However, after a long period of follow-up and/or after clinical tests, the control cases were finally diagnosed with a non-gastrointestinal disorder, often psychosomatic in origin.

The diagnosis of CMSE in this study was based on positive reaction in an open oral antigen challenge with increasing doses. In Finland, the DBPCFC test performed at ward is nowadays considered as a too expensive and unpractical way of confirming milk-related symptoms (Duodecim 2004). Single-blind food challenge and open food challenge are the mostly used techniques but with the open test placebo effect can always confuse the symptom diary. Also with the DBPCFC test, the rate of false negative diagnosis is about 3% because of unsatisfied time of milk challenge (Caffarelli & Petroccione 2001). But if there is a history of acute reactions related to milk or suspicious for IgE-mediated mechanisms, the possibility of severe reactions should always be kept in mind (David 1984) and these patients are challenged at hospital.

7.2 Methods

Since histological analyses are prone to subjectivity, all analyses were performed blindly, without clinical or endoscopic data. Some disagreement between observers is common in all histo-pathological evaluations including assessment of duodenal villous atrophy (Weile et al. 2000). Therefore, each histological and immunohistochemical analysis was performed by a single observer. Treatment of biopsy specimens, such as fixation and embedding may affect immunoreactivity. Therefore, all specimens were prepared in a single laboratory with a consistent, monitored routine.

We designed a new cell counting system to provide regional data about the location of CTLs and NKs in different parts of the villi. Using a light microscope and a 100x objective with oil immersion to provide detailed distinction, cell counting was performed in every optimally cut half-villus unit where a well-orientated villus could be observed for its whole length. By using a simple spread sheet program with a conventional computer, we were able to count cell numbers and densities in different parts of the villi. Since IELs in CD show characteristic distributional changes (Biagi et al. 2004, Jarvinen et al. 2003a), we found it important to analyse epithelial IEL distribution in CMSE.
In normal duodenum, the GrA was the most prevalent cytotoxic protein, the number of positive IELs were about 60% of the CD3⁺ IELs, while for GrB the proportion was less than 15%. These figures are higher than those in previous reports where approximately 30% of IELs have expressed GrA and less than 2%, GrB (Chott et al. 1997). In our control cases, PFN counts were also higher (50% of CD3⁺ IELs) than previously reported (Ciccocioppo et al. 2000, Chott et al. 1997, Melgar et al. 2002). These discrepancies may be related to either a different age distribution of cases (children vs. adults), or methods such as the antibody used or the sensitivity of the detection system. We used paraffin sections, in which morphology is better preserved than in frozen sections, and may be better suited to demonstrating mild focal immunoreactivity. In addition, we used a highly sensitive detection system EnVision (Sabattini et al. 1998) and the light microscope analysis was done at a very high magnification, all these methodological issues contributing to higher sensitivity in detecting positive cells.

7.3 Immunopathogenesis of CMSE

From previous reports of a dominant Th1 cytokine profile (Hauer et al. 1997) and the results of this thesis, cell mediated hypersensitivity to cow’s milk proteins seems to be the allergic mechanism behind CMSE. Our study shows that cytotoxic activation of IELs is characteristic of CMSE. There is still a lack of knowledge of the significance of the increased densities of CD3⁺, γδ-TCR and cytotoxic granule bearing IELs in CMSE. In CD, the precise etiology has been determined in detail but the immunological or other mechanism remains unknown in CMSE. The strongest descending duodenal mucosal immunoreactivity in CMSE cases was equivalent to that in the milder CD cases, but no villous shortening was seen. In the early phase of CD, the sole morphological finding is an increase of CD3⁺ IELs (Jarvinen et al. 2004), a feature identical to CMSE. It seems likely that CD starts with immunological activity that has some features in common with CMSE. Compared to CD or CMP, histological changes on routine sections were minimal or absent and no sign of villous atrophy was seen, suggesting that either the pathogenetic mechanisms, the intensity of the reaction or both are different to CMSE.

The relationship between the cell-mediated immune response and the symptoms of CMSE is not clear. The mucosal autonomous nervous system might be sensitized by cytotoxic cells and mucosal mediators. Could the function of the descending duodenal epithelium be disrupted resulting in permeability changes etc? The rate of activation induced cell death (AICD) in CMSE needs further functional studies. If the rate was shown to be increased, the phenomenon would serve as a protective mechanism against cytotoxicity, preventing mucosal damage. This area would be a useful avenue of future investigation.

7.4 Cytotoxic granules in duodenal IELs

In both CD and CMSE, the numbers of γδ-TCR⁺, CD3⁺, TIA-1, PFN and GrA containing IELs were increased, and in CD the GrB expression as well, supporting the importance of
deranged cell mediated immunity involving lymphocyte cytotoxicity. Although the absolute densities of TIA-1, GrA, and PFN expressing cells in the descending duodenum were increased in CMSE compared with controls, the proportions of CD3+ IELs were similar. Whether this can be explained by an increased number of intraepithelial NK cells, the activation of CD3+ IELs or both needs further study.

We found evidence indicating that abnormal cytotoxicity in CMSE is related to antigen exposure and is not a constant abnormality in these subjects. The numbers of TIA-1 expressing lymphocytes were significantly reduced in patients on an elimination diet, although there was some overlap in the counts. In addition, our analysis of serum granzymes showed decreased concentration in treated CMSE patients, supporting the theory of abnormal cytotoxicity caused by cow’s milk.

The basic pattern of cytotoxic activation in CMSE seems to be generally similar to CD, but there are differences in the anatomical distribution and intensity of cytotoxicity, suggesting that there are differences in the initiating mechanisms and the regulatory processes. In CD, the most severe villous changes are located in the most proximal parts of the small intestine (Oberhuber 2000), possibly related to the higher concentration of 33-mer gliadin peptides in this area. For largely unknown reasons, the distribution of lesions is wider and more variable in CMSE (Kokkonen et al. 2001). This is possibly related to the degree of digestion of antigenic peptides or proteins. T cell epitopes are often short peptides, which might explain the location of the lesions in the distal part of small intestine. Consistent with this idea, we showed that in CMSE, the cytotoxic response is increased in the descending part of the duodenum, but not in the bulbus.

The preferential distribution of IELs in CMSE mimics the normal distribution of IELs, but with a somewhat higher density in the villus tip region. Recently it has been suggested that increased IEL counts in the villus tip region are useful in the diagnosis of CD even in the absence of villous abnormality (Biagi et al. 2004, Goldstein & Underhill 2001). Our observations indicate that this kind of alteration is not specific for CD; CMSE has to be considered in the differential diagnosis when an increase of IELs in the villus tip region is found.

In CD, our results were in agreement with previous reports of increase in GrB and PFN density (Oberhuber et al. 1996, Cicciocippo et al. 2000). Instead, a novel observation was a significant increase of GrA expression the counts of GrA positive cells being on average more than 90% of the counts of CD3+ IELs. Since the proportions of CD3+ and NKs marker positive IELs are low in active CD, with the majority of IELs being CD3+ T cells (Spencer et al. 1989b, Camarero et al. 2000), we may conclude that in CD, a majority of intraepithelial CD3+ T lymphocytes express GrA, GrB, and PFN. Since GrA and GrB induce apoptosis independently and synergistically in a PFN-dependent manner (Lieberman 2003), this machinery provides an effective cytotoxic mechanism in CD. In CMSE, the absence of increase of GrB expressing cells may be related to less effective cytotoxicity in spite of an increase in GrA and PFN expressing cells. Although not systematically assessed, the extent of immunoreactivity of cytotoxic granules seemed to show a disease specific pattern. In the control and CMSE samples, positive granules were usually small, while in CD cases, cytoplasm of IELs was usually filled with immunoreactive granules. This suggests that in addition to the number of positive cells, the quantity of cytotoxic molecules may be an important indicator of actual cytotoxic activity.
7.4.1 Apoptosis and proliferation

The TUNEL method is able to detect cells undergoing programmed cell death at an early stage by labelling the 3'-ends of DNA exposed by histone degradation. This technique has been shown to be a more sensitive method of recognizing apoptotic cells than counting the apoptotic bodies by routine microscopy (Gavrieli et al. 1992). The monoclonal antibody M30 recognizes an epitope of cytokeratin 18 after an early caspase cleavage during apoptosis, and is considered a specific and early detector of apoptotic epithelial cells (Leers et al. 1999). It was recently suggested that there is only a small amount of cytokeratin 18 in the proximal human intestine, possibly making M30 staining unsuitable for identifying apoptotic epithelial cells in this location (Groos et al. 2003). However, we consistently found M30+ cells in our intestinal samples, but were not able to demonstrate any increase in enterocyte apoptosis with the M30 antibody in CD. Consistent with the previous data on the elimination of apoptotic enterocytes in the intestinal lumen (Madara 1990), M30 immunoreactivity of LP was practically absent. In CMSE, an increase in epithelial TUNEL+ cells was seen in the descending duodenum, located in the villous tips and mid portions of the villus. This observation slightly strengthens the idea of lymphoid but not enterocyte apoptosis, that is thought to occur in the villus tip region.

The varied symptoms of CMSE and CD seem to be related more to functional imbalances in the immune system, than to morphological changes. As we know, there is a non-symptomatic form of CD that nonetheless demonstrates mucosal damage with villus atrophy and excess of IELs. In CMSE, no signs of villous atrophy or crypt hyperplasia were seen suggesting that there is neither significant loss of epithelial cells nor compensatory epithelial proliferation. The reported increased rate of proliferation in cow’s milk protein intolerance of young infants is likely to be related to the presence of villous atrophic changes and more severe disease in these patients (Kosnai et al. 1980, Savidge et al. 1996). We were not able to study the effect of elimination treatment on the apoptosis counts, but our previous observations indicate that antigen elimination is associated with a decreased density of cytotoxicity (I, IV). This would suggest that increased apoptosis is similarly an antigen related phenomenon and not a constitutional defect.

Normal rates of enterocyte apoptosis or proliferation rate but an increased rate of epithelial TUNEL apoptosis supports the idea that apoptosis involves only lymphoid cells (III). The increased apoptosis might be a compensatory mechanism of maintaining homeostasis and reducing the numbers of IELs. We speculate that increased apoptosis might be a manifestation of a process known as activation-induced cell death, where a repeated stimulation of T cell receptor sensitizes activated T cells to apoptosis. Although no association was found between granule and TUNEL indices in the CMSE group perhaps the number of cytotoxic granules does not correlate with the rate of lymphocyte apoptosis. We studied serum sFas concentration but observed no increase in any group or any influence of a milk elimination diet on serum sFas levels. Activation induced cell death of CTLs is a highly controlled mechanism of self-regulation mediated by Fas/FasL interactions, and may require functional assays to be studied further.
We were also able to show that there is increased density of TUNEL$^+$ cells in the villous LP of CMSE patients. Increased apoptosis of the cells of the mucosal immune system might be part of a mechanism attempting to achieve tolerance or a compensatory mechanism to retain homeostasis. The density of apoptotic cells in the LP correlated positively with the epithelial TUNEL counts in both CD patients and controls, indicating a regulatory relationship between these two tissue compartments. However, no such correlation was seen in CMSE subjects, suggesting disturbance of such a homeostatic mechanism in these patients.

Only in the control cases was there a significant difference in the TUNEL counts between the bulb and descending duodenum, the indices of the bulb being higher. We speculate that in the absence of mucosal immunological activation, some non-immunological local factor, such as higher luminal acidity, becomes apparent. Within the CMSE group, there was a trend of increased apoptosis in the descending duodenum compared to the bulb. One could speculate that perhaps this is related to more advanced proteolytic degradation in this location, allowing small milk epitopes not yet available in the bulb to induce immunological activation and an increased apoptosis rate.

7.4.2 Serum responses

Concentrations of GrA and GrB were significantly higher in untreated CMSE and in CD than in the controls. The significant correlation between the serum granzyme levels and the numbers of duodenal intraepithelial CD3$^+$, $\alpha\beta^+$- and $\gamma\delta^+$ TCRs strongly suggests that serum concentrations are indeed related to mucosal immunological activation and actual cytotoxic activation in the intestinal mucosa. In CMSE, we have recently found some evidence for a correlation between GrA expressing cell density in duodenal LP and the serum GrA concentration (unpublished observations). This suggests that serum concentrations are indeed related to actual cytotoxic activation in the intestine. Our recent finding that even GrA expressing cells in the LP are increased in CMSE (unpublished observations) supports the idea that cytotoxic activation is present both in the epithelium and the LP. The increase in the LP, where there is a rich capillary network, might be a more plausible origin for the increased serum levels than IELS, which have no such relationship with blood vessels.

An interesting and unexpected finding was that the GrB level in CMSE subjects was higher than in CD subjects, even though the level is supposed to have a direct correlation with the density of IELs and cytotoxic activity in the small intestinal mucosa, which is higher in CD subjects and far lower in CMSE patients. In CMSE the distribution of mucosal changes varies and in many cases; in addition to the abnormalities in the proximal small intestine, ileal and colon mucosa may also show an increase of IELs (Kokkonen et al. 1999). We therefore speculate that, as the extent of villous changes in CD are associated with serum GrA levels, the serum concentration of GrB in CMSE may depend on the extent of the mucosal lesions; higher values possibly associating with a more extensive intestinal immune response. However, more studies are necessary to confirm this idea.
Dominance of Th1 cytokine profile has been observed in CMSE (Hauer et al. 1997). We found the serum CD30 to be significantly increased in both CMSE and CD, but there was much overlap with the controls, indicating a less discriminative value compared with the granzyme assays (IV). Previously, the expression of CD30 was thought to be restricted in activated T cells producing Th2-type cytokines (Romagnani et al. 1995). However, recent results of functional studies have suggested give that CD30 may be an important costimulatory molecule and marker for the physiological balance between the TH1 and TH2 immune response (Pellegrini et al. 2003). In the duodenal mucosa, CD30+ cells are barely detectable in patients with uncomplicated CD but patients with refractory sprue, a form of CD not responding to a gluten free diet, have phenotypically abnormal IELs that lack CD8, αβ-TCR or γδ-TCR, and/or express CD30 in addition to variable expression of the NK cell receptor CD94. Farstad et al concluded that abnormal CD30 expression may indicate a worse prognosis, including the occurrence of overt lymphoma. (Farstad et al. 2002) Further studies are needed to see whether high serum CD30 concentration would serve as a marker for refractory sprue and the related lymphoma.

All our groups showed similar serum sFas concentrations. Cheng et al. (1994) found that sFas blocked apoptosis induced by the antibody to Fas through the mechanism of inhibiting the interaction between cell surface Fas and FasL in vitro, and altered lymphocyte development and proliferation in response to self antigen (Cheng et al. 1994). In active ulcerative colitis, in which cell mediated immune responses are acknowledged, serum sFas was significantly decreased but on immunohistochemistry, the number of colonic FasL+ and TUNEL+ cells was significantly higher. The workers raised a hypothesis that perhaps a decrease in local sFas, which is directly affected by a decrease in serum sFas level, promotes apoptosis in inflamed colonic mucosa of ulcerative colitis (Yukawa et al. 2002).

### 7.5 Diagnostic aspects

The mild macroscopic abnormalities and traditional histo-pathological changes we found in our CMSE patients were not specific, indicating that the main role of basic histopathology is the exclusion of diseases such as CD and not in the positive identification of a patient with gastrointestinal milk hypersensitivity. Endoscopic abnormalities such as LNH were more prevalent, but occurred in a significant proportion of patients in other groups as well. It may be concluded, that CMSE should be included in the differential diagnostic consideration when an increase in IELs, a hallmark of CD (Oberhuber 2000) is seen in biopsies, or LNH is observed at endoscopy. Either LNH or an increased number of IELs or their combination is specific enough to diagnose CMSE. However, an elimination-challenge test may be indicated.

When intestinal biopsies are taken to investigate the cause of prolonged gastrointestinal symptoms, immunohistochemistry for TIA-1, PFN and GrA may provide some diagnostically useful information. Although the differences in the indices of descending duodenum between CMSE and control cases were significant, there was some overlap (Table 5) within the groups indicating wide individual variation. Based on the ratios presented in Table 5, especially the numbers of TIA-1 (2.8-fold increase), PFN
(2.0-fold increase), and GrA (2.0-fold increase) positive IELs in lower villous epithelium seem to serve as the most distinctive markers in CMSE. With normal villous architecture but positive CD serology, noticeable expression of cytotoxic IELs especially in the villous tip, can be an early finding, before the development of villous atrophy.

Our results indicate that in cell-mediated food hypersensitivity such as CMSE the measurement of serum granzymes might provide a novel, non-invasive way of monitoring immune-mediated intestinal diseases both during the initial diagnosis and in the assessment of treatment response. However, it is apparent that the increment in serum granzyme concentrations as such has no specific relation to the aetiological factor inducing lymphocyte activation; increased serum granzyme levels have been detected in viral (Spaeny-Dekking et al. 1998), parasitic (Hermsen et al. 2003), bacterial infections (Lauw et al. 2000) and in autoimmune diseases such as scleroderma (Kahaleh & Fan 1997) and rheumatoid arthritis (Spaeny-Dekking et al. 1998). We feel that serological markers of cell-mediated immune response in non-IgE-mediated food hypersensitivity merit further study.

In CD, serum GrA or GrB determination is probably of no use in the primary diagnosis. However, granzyme levels showed an association with the extent of villous atrophy, association with GrA being statistically significant. These findings suggest that granzyme determinations might have some value in CD as a complementary screening test, increased levels suggesting mucosal immune activation of probable clinical significance, and thus helping to determine the need for additional or follow-up investigations in cases with borderline or inconsistent results in the standard CD screening tests. Further studies are also needed to see whether a demonstration of significant decrease in these immune activation markers could be applied in assessing treatment response in CD.

### 7.6 Future aspects

Our series of CMSE patients had been sent to a specialist referral centre and therefore probably represent the more severe end of the disease spectrum. The value of serum granzyme assays has to be assessed in a series with mild symptoms before their use can be recommended. Elimination-challenge tests are necessary for the diagnosis of even mild cases. Symptomatic response to an elimination-challenge test may take several weeks, but a significant fall or increase of serum granzymes might occur more rapidly and provide a more objective measure of the response. Monitoring of the serum granzyme levels could optimize the duration of elimination and challenge periods. Most importantly, further studies are needed to investigate the pathological mechanisms linking milk antigens with the morphological and clinical features of CMSE.

Cytotoxicity mechanisms are important in the destruction of cells and tissues in autoimmune diseases (Russell & Ley 2002). In type 1 diabetes, beta cell death ultimately appears to be caused by Fas/FasL-mediated mechanisms and/or by secretion of granzymes and PFN (Kawasaki et al. 2004). Savilahti with his co-workers reported of newly diagnosed diabetes patients with increased concentrations of antibodies to cow’s milk proteins (Paronen et al. 2000). An increased incidence of separate autoimmune
disorders was found in a group of adolescents with infantile atopic eczema (Kokkonen & Niinimaki 2004). The same effect was earlier described by Ventura et al. (2000) studying CD patients (Ventura et al. 2000). It is possible that activated immune responses, perhaps early in childhood, may predispose people to later autoimmune disorders by an unknown mechanism. As the number of subjects sensitized for cow’s milk is numerous compared to subjects reacting to gliadin, the effect of milk sensitization may be even more important.
8 Conclusions

This study evaluated the expression of cytotoxicity and apoptosis related immunohistochemical and serological markers in children with CMSE, comparing them with findings in CD. Our observations show that cell mediated allergic mechanisms are involved in CMSE, and that the up-regulated immune response in the intestinal mucosa is characterized by an increased expression of cytotoxic granules, and that the intensity of reaction is milder than in CD. This cytotoxic activation can be assessed by determining serum GrA and GrB concentrations. Our finding introduces a potential means for the diagnostic assessment of these diseases. We believe it is possible to develop a more specific and shorter elimination-challenge test based on the decline in serum markers of the cytotoxic immune response during the elimination period and their rise during the challenge. However, we still need more comprehensive studies to determine the detailed association between these markers and the skewing of the mucosal lymphoid system. Based on the results presented on this thesis, the main conclusions are:

1. The expression of TIA-1 was increased in lymphoid cells in the epithelial compartment of the descending duodenum in children with CMSE. The same phenomenon was not observed in the antrum or the bulb. The TIA-1 expression of the descending duodenum was decreased during an elimination diet. In CD, increased TIA-1 expression was seen both in the bulbus and the descending duodenum.

2. The IEL expression of PFN and GrA but not that of GrB was increased in the mucosal samples of descending duodenum in children with CMSE. A similar increase was not observed in the bulb. In CD, all three markers showed strong expression in both regions.

3. In both CMSE and in CD, the rates of epithelial and villous LP apoptosis, detected by the TUNEL technique, were increased in the descending duodenum. A similar increase was not observed in the bulb. The densities of apoptotic epithelial cells detected by M30 antibody were similar in both groups. There was a difference in proliferation rate in CD, but not in CMSE.

4. The serum concentrations of GrA, GrB and CD30 were elevated in CMSE and in CD groups, the increase being stronger in CMSE. The concentrations of treated CMSE subjects and CD cases with milder villus atrophy were lower.


References


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