Serum Antibodies in Coeliac Disease

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SATU SULKANEN

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ACADEMIC DISSERTATION

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ACADEMIC DISSERTATION

To be presented, with the permission of
the Faculty of Medicine of the University of Tampere,
for public discussion in the auditorium of
Finn-Medi, Lenkkeilijänkatu 6, Tampere
on 3 March 2000 at 12 o'clock.

University of Tampere
Tampere 2000

To Jari, Emmiina, Adeliina and Aleksi

Contents

List of original publications

This thesis is based on the following original communications, referred to in the text by their Roman numerals I-IV:

I Sulkanen S, Halttunen T, Marttinen A, Leivo E-L, Laurila K and Mäki M (1998): Autoantibodies in celiac disease: importance of fibroblasts. J Pediatr Gastroenterol Nutr 27:206-213.

II Sulkanen S, Collin P, Laurila K and Mäki M (1998): IgA- and IgG-class antihuman umbilical cord antibody tests in adult coeliac disease. Scand J Gastroenterol 33:251-254.

III Sulkanen S, Halttunen T, Laurila K, Kolho K-L, Korponay-Szabó IR, Sarnesto A, Savilahti E, Collin P and Mäki M (1998): Tissue transglutaminase autoantibody enzyme-linked immunosorbent assay in detecting celiac disease. Gastroenterology 115:1322-1328.

IV Korponay-Szabó IR, Sulkanen S, Halttunen T, Maurano P, Rossi M, Mazzarella G, Laurila K, Troncone R and Mäki M: Tissue transglutaminase is the target in both rodent and primate tissues for coeliac disease-specific autoantibodies. J Pediatr Gastroenterol Nutr, accepted.

Abbreviations

AGA	gliadin antibody
ARA	reticulin antibody
AU	arbitrary units
CD	cluster design
ELISA	enzyme-linked immunosorbent assay
EmA	endomysial antibody
ESPGAN	European Society for Paediatric Gastroenterology and Nutrition
FITC	fluorescein isothiocyanate
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
Ig	immunoglobulin
kD	kilodalton
KSCN	potassium thiocyanate
mAb	monoclonal antibody
Np_{od}	optical density of the negative control serum pool
PBS	phosphate-buffered saline
S_{od}	optical density of the sample under analysis
TBS	Tris-buffered saline
TCR	T cell receptor
TRITC	tetra-methyl-rhodamine isothiocyanate isomer R
TTBS	Tween-20 in TBS
tTG	tissue transglutaminase

Introduction

Coeliac disease is defined as a permanent intolerance of the small-bowel mucosa to dietary wheat gliadin or related proteins in cereals, resulting in intestinal lesions in genetically susceptible individuals. The exact mechanisms by which ingestion of gluten causes mucosal damage are still unknown, but evidence suggests that T-cell mediated activating process plays an important role in the pathogenesis of this disorder (MacDonald and Spencer 1988, Lundin et al. 1993). A definitive diagnosis can be obtained only by histopathological demonstration of small-bowel mucosal villous atrophy and crypt hyperplasia. Treatment with a gluten-free diet results in clinical recovery and improvement in histological appearance. Further, the possible risk of complications associated with untreated coeliac disease, for example osteopenia (Valdimarsson et al. 1996a), liver involvement (Volta et al. 1998), idiopathic ataxia (Hadjivassiliou et al. 1998) and small-bowel lymphoma (Holmes et al. 1989), can be reduced by early adoption of a gluten-free diet.

The prevalence of coeliac disease is possibly as high as 1% in the general population (Johnston et al. 1997), and the clinical picture of the condition is heterogenous (Mäki and Collin 1997). Manifestations are quite frequently atypical, and the disease can even be symptom-free (Logan et al. 1983, Collin et al. 1990). There is thus, a need for simple, reliable and cost-effective non-invasive screening tests to ensure that clinically mild forms of the disease with uncharacteristic symptoms are not overlooked. To this end, interest has focused on serologic investigations and serum gliadin antibody (AGA) tests by enzyme-linked immunosorbent assays (ELISA) are widely used in screening (Troncone and Ferguson 1991). Unfortunately, however, false positive AGA titres are found in healthy individuals and in patients affected by other diseases. Serum reticulin

antibodies (ARA), determined by immunofluorescence with rat liver and kidney as substrate, have been in use since 1971 (Seah et al. 1971). In 1983, Chorzelski et al. described a new group of tissue antibodies, endomysial antibodies (EmA), directed against the intermyofibrillar substance of the monkey oesophagus smooth muscle. The immunoglobulin (Ig)A-class R_1-type ARA and EmA have been found reliable and valuable in early recognition of untreated coeliac disease (Mäki et al. 1984a, Hällström 1989, Volta et al. 1991). Recently, there has been interest in the use of human umbilical cord as a substitute for the oesophagus of an endangered species (Ladinser et al. 1994).

Gluten-induced ARA and EmA are tissue autoantibodies which have been shown to be directed against extracellular matrix non-collagenous proteins produced by fibroblasts (Mäki et al. 1991a, Marttinen and Mäki 1993). Recently, Dieterich et al. (1997) identified tissue transglutaminase (tTG) as the antigen recognised by EmA. tTG belongs to a family of calcium-dependent enzymes which catalyse the cross-linking of proteins by introducing a covalent bond between lysine and glutamine residues. Dieterich et al. (1997) showed that coeliac disease serum IgA reacts with tTG, suggesting that the detection of IgA-class autoantibodies against tTG might be useful in coeliac disease diagnosis.

The purpose of the present study was to assess the value of human umbilical cord and human umbilical cord Wharton's jelly-derived fibroblasts as antigens in screening tests for untreated coeliac disease. Further, it was sought to establish whether IgA-class tTG autoantibodies can be considered specific for coeliac disease. The tissue distribution of tTG was analysed to clarify the relationship between ARA, EmA and tTG antibodies.

Review of the literature

Clinical aspects of coeliac disease

Epidemiology

The prevalence of clinically manifest coeliac disease has been estimated to be about 1:1000. Regional differences have, however, been reported. A multicentre European survey on the frequency of identified cases in childhood reported rates from 1 in 250 (observed in Sweden) to 1 in 4000 (in Denmark) (Greco et al. 1992). In recent years, population screening studies have suggested that the prevalence of the condition is substantially higher than supposed. Grodzinsky et al. (1992) reported a prevalence of one in 250 in Sweden upon screening blood donors with AGA. A similarly high prevalence was found in Italy in screening a group of schoolchildren for coeliac disease with AGA and EmA (Catassi et al. 1994). In Finland a prevalence of 1:370 was found by frequently taking small-bowel specimens and focusing serological screening on risk groups (Collin et al. 1997). Recent screening studies have suggested a true prevalence possibly even higher, with estimated rates of one in 122 for Ireland, one in 130 for Finland, one in 100 for Italy, one in 198 for the Netherlands and one in 85 for Hungary (Johnston et al. 1997, Kolho et al. 1998, Csizmadia et al. 1999, Meloni et al. 1999, Korponay-Szabo et al. 1999). In the USA diagnosis of coeliac disease has remained quite rare (Fasano 1996). However, a high prevalence of EmA in blood donors (Not et al. 1998), and the occurrence of silent coeliac disease among patients with type I diabetes (Rossi et al. 1993, Talal et al. 1997, Fraser-Reynolds et al. 1998) indicates that the clinical situation in the USA might well be similar to that in Europe.

Previous epidemiological studies have suggested significant variations in the prevalence of coeliac disease by time. In the late 1970s the frequency of coeliac disease diagnosis seemed to be on the decline especially in children under two years of age, indicating that childhood coeliac disease is disappearing (Challacombe and Bayliss 1980, Littlewood et al. 1980, Dossetor et al. 1981). In Finland the decrease was also observed, but was found to be at least in part due to an upward shift in age at diagnosis (Mäki et al. 1988a). In Sweden, in contrast to other European countries, a threefold increase has been reported in the incidence of the disease manifesting in infancy and at early ages (Ascher et al. 1991). This is probably attributable to a high gluten consumption in infancy (Cavell et al. 1992, Mäki et al. 1992).

Clinical picture

Classical symptoms of coeliac disease in children include abdominal distension, diarrhoea, steatorrhoea, vomiting and failure to thrive. These are likely in infants less than two years of age (Visakorpi et al. 1967). In older children and adolescents gastrointestinal symptoms are less common (Visakorpi et al. 1970). Recent years have seen a significant decrease in symptomatic infantile coeliac disease (Littlewood et al. 1980, Dossetor et al. 1981, Kokkonen et al. 1982, Stevens et al. 1987). First manifestations have shifted to later ages, the clinical presentation being simultaneously altered (Mäki et al. 1988b). Classic symptoms have become rare, gastrointestinal symptoms are milder and often even absent, and coeliac disease without abdominal symptoms can be found in children with short stature, iron deficiency anaemia, delayed puberty and joint complaints (Verkasalo et al. 1978, Stenhammar et al. 1986, Mäki et al. 1988a, Mäki et al. 1988b).

Adults may present with coeliac disease at any age. In many clinically overt cases dyspepsia, abdominal bloating and diarrhoea may be involved (Cooke and Holmes

1984, Kelly et al. 1990). However, as in the case of children a shift of the disease toward subtle and variable clinical forms has also been observed in adults (Swinson and Levi 1980, Logan et al. 1983, Collin et al. 1990, Corazza et al. 1993).

In recent years, coeliac disease patients with more or less silent presentation are being diagnosed at an increasing rate – for example in family studies. Despite the manifest mucosal lesion, affected individuals may have only mild or no symptoms and signs of coeliac disease (Ferguson et al. 1993, Visakorpi and Mäki 1994).

Diagnostic criteria

When the first precise clinical description of coeliac disease was published by Samuel Gee in 1888, the diagnosis was based entirely on the detection of clinical features. In the 1950s the discovery of small-bowel villous atrophy reacting to a gluten-free diet and the advent of the peroral small-bowel biopsy technique made more accurate coeliac disease diagnosis possible. The first diagnostic criteria for coeliac disease as stated by the European Society for Paediatric Gastroenterology and Nutrition (ESPGAN) in 1969, included the finding of a structurally abnormal small-bowel mucosa when the patient is eating cereals, a clear improvement in villous structure on a gluten-free diet, and deterioration of the mucosa upon gluten challenge (Weijers et al. 1970). In 1990, these criteria were reconsidered (Walker-Smith et al. 1990). The appearance of characteristic small-bowel mucosal atrophy and clinical remission on a gluten-free diet remain essential without further biopsy confirmation. In asymptomatic patients, however, a second biopsy sample is necessary to prove mucosal recovery on treatment with a gluten-free diet. In cases of doubt as to the initial diagnosis, e.g. in children younger than two years living in communities where other causes of enteropathy occur, or in patients in whom the findings in their first biopsy sample are equivocal, gluten challenge is called for. The presence of circulating antibodies, i.e. AGA, ARA and

EmA, at the time of diagnosis in patients with a typical small-intestinal mucosa, and their disappearance in parallel to a clinical response to a gluten-free diet, strongly supports the diagnosis of coeliac disease.

In recent years it has been increasingly recognised that gluten sensitivity is no longer present exclusively in patients envincing villous atrophy on a gluten-containing diet. Observations of the intestinal response to gluten have revealed a continuum of histological features, with the flat lesion at one end of the spectrum and a normal mucosal villous and crypt architecture, but an abnormally high density or count of intraepithelial lymphocytes at the other (Marsh 1992, Ferguson et al. 1993). Individuals yielding a normal jejunal biopsy while on a normal diet may have latent coeliac disease, where overt coeliac lesion develops at some other time and the mucosa recovers on a gluten-free diet (Weinstein 1974, Ferguson et al. 1987, Mäki et al. 1990, Troncone 1995, Corazza et al. 1996). A high count of villous intraepithelial lymphocytes (Ferguson and Murray 1971), increased γδ+ T-cell receptor (TCR) expression by intraepithelial lymphocytes (Mäki et al. 1991b), abnormal jejunal permeability (Bjarnason et al. 1983), a positive coeliac-like intestinal antibody pattern (O'Mahony et al. 1991, Arranz and Ferguson 1993) and serum ARA/EmA positivity (Collin et al. 1993, Kaukinen et al. 1998) have been considered to be markers of coeliac disease latency.

Manifestations, complications and associations of coeliac disease

Dermatitis herpetiformis, a gluten-sensitive blistering skin disease characterised by pathognomic granular IgA deposits in the uninvolved skin, is a classical example of the extraintestinal manifestations of coeliac disease (Reunala et al. 1984). Reunala et al. (1984) reported that although symptoms suggesting small-intestinal disease were rare,

approximately 75% of patients with dermatitis herpetiformis had small-bowel villous atrophy with crypt hyperplasia. Permanent-tooth enamel defects (Aine et al. 1990), as well as neurological disorders such as idiopathic ataxia (Hadjivassiliou et al. 1998) and epilepsy with posterior cerebral calcifications (Collin et al. 1991, Gobbi et al. 1990) can also be regarded as gluten-induced extraintestinal manifestations of the disease.

Many subjects with untreated coeliac disease have been reported to show liver involvement (Jacobsen et al. 1990, Bardella et al. 1995, Volta et al. 1998). Volta et al. (1998) found that about 9% of patients with hypertransaminasaemia of unknown, cryptogenic, origin are affected by symptom-free coeliac disease. Serum transaminase concentrations returned to normal levels after a few months of a strict gluten-free diet. It had also previously been demonstrated that symptomatic coeliac disease patients suffer from osteopenia which recovers on a gluten-free diet (Mazure et al. 1994, Valdimarsson et al. 1996a). A recent study showed osteopenia in 19 individuals whose clinically silent coeliac disease was detected by active screening only; bone mineral density clearly increased under dietary treatment (Mustalahti et al. 1999). In addition, a strict gluten-free diet seems to protect against the development of the small-intestinal lymphomas reported to complicate coeliac disease (Holmes et al. 1989, Collin et al. 1994a). Also recurrent abortions, infertility (Sher et al. 1994, Collin et al. 1996), and aphtosis of the oral mucosa (Ferguson et al. 1980) may be associated conditions.

The occurrence of autoimmune diseases in patients with coeliac disease is well established (Lancaster-Smith et al. 1974, Cooper et al. 1978, Snook et al. 1989, Collin and Mäki 1994). Further, a recent study showed that the prevalence of autoimmune disorders in coeliac disease is related to the duration of exposure to gluten (Ventura et al. 1999). Type I diabetes is one such organ-specific autoimmune disorder described in this context; the prevalence of coeliac disease in children with type I diabetes has been

between 2.3% and 4.8% (Mäki et al. 1984b, Savilahti et al. 1986, Rossi et al. 1993, Acerini et al. 1998) and in adults with type I diabetes between 2% and 6.4% (Collin et al. 1989, Page et al. 1994, Sategna-Guidetti et al. 1994, Rensch et al. 1996, Cronin and Shanahan 1997). Coeliac disease also occurs with increased frequency in patients with autoimmune thyroid disease (Collin et al. 1994b), and has been connected with alopecia areata (Corazza et al. 1995) and the relatively uncommon Addison's disease (Reunala et al. 1987, Collin et al. 1994a). Connective tissue disorders associated with coeliac disease include juvenile rheumatoid arthritis (Mäki et al. 1988b) and Sjögren's syndrome (Collin et al. 1994a, Kerttula et al. 1996, Iltanen et al. 1999a).

Sarcoidosis (Douglas et al. 1984), atopy and asthma (Hodgson et al. 1976, Cooper et al. 1978) are other disorders reported to occur concomitantly with coeliac disease. Furthermore, patients with selective IgA deficiency are at an increased risk of coeliac disease (Savilahti et al. 1971, Collin et al. 1992a, Cataldo et al. 1998), and there is also convincing evidence for an association between coeliac disease and Down's syndrome (Similä and Kokkonen 1990, George et al. 1996, Gale et al. 1997).

Treatment

It is generally accepted that the major triggering factors in coeliac disease are the prolamins of wheat (gliadin), rye (secalin) and barley (hordein). All these fractions are considered to be toxic for coeliac patients (Howdle et al. 1984, Sturgess et al. 1991) and should be withdrawn from the diet. Recent studies have suggested that oats may safely be consumed by patients with coeliac disease (Janatuinen et al. 1995, Srinivasan et al. 1996) and dermatitis herpetiformis (Hardman et al. 1997, Reunala et al. 1998). Kaukinen et al. (1999) concluded that wheat-starch-based gluten-free products were not harmful in the treatment of coeliac disease and dermatitis herpetiformis.

In clinical practice compliance with the gluten-free diet may be far from satisfactory. Kumar et al. (1988) reported strict adherence to a gluten-free diet in 57% of teenagers, and Bardella and coworkers (1994) found that only 45% of their adult patients maintained a strict diet. However, many patients appear to feel well and lack symptoms following a normal gluten-containing diet (Kumar et al. 1988, Mäki et al. 1989, Mayer et al. 1991). A regular follow-up seems to correlate positively with dietary compliance, which would imply that lifelong monitoring is essential in the management of coeliac patients (Bardella et al. 1994).

Genetic factors and pathogenetic aspects of coeliac disease

Genetic background

Familial clustering of coeliac disease is evidenced by its increased prevalence in relatives of patients; in family studies the disease frequency among first-degree relatives of probands has been found to be about 10% (Mylotte et al. 1974, Auricchio et al. 1988, Mäki et al. 1991c, Holm 1993, Petronzelli et al. 1997). The concordance rate approximately 70% reported among monozycotic twins constitutes additional support for the conception of an inherited predisposition to the disorder (Polanco et al. 1981).

Certain genes within the HLA gene complex, found on the short arm of chromosome 6, appear to confer susceptibility to coeliac disease (Sollid and Thorsby 1993). Coeliac disease was first observed to be associated with the HLA class I molecule B8 (Falchuk et al. 1972, Stokes et al. 1972), and firmer associations with the class II allele DR3 (Keuning et al. 1976, Ek et al. 1978), and also with DR7 and DR5/DR7 (DeMarchi et al. 1983, Mearin et al. 1983) were subsequently revealed. Among Finnish coeliacs the frequency of DR3 antigen was 92% (Mäki et al. 1991c), 59% among family members of coeliac patients and only 23% in healthy control subjects (Holm 1993). An even

stronger association has since been found with the DQ2 allele (Tosi et al. 1983), and it has been suggested that the primary association of coeliac disease is in fact with HLA-DQ2, i.e. the DQ α/β heterodimer, encoded by the alleles DQA1*0501 and DQB1*0201 located either in *cis* (in DR3-DQ2-positive individuals) or in *trans* position (in DR5/7-DQ2 heterozygous individuals) (Sollid et al. 1989). This configuration is expressed by 95% of coeliac disease patients as against 20-30% of controls (Tosi et al. 1983, Sollid et al. 1989, Ploski et al. 1993). In Finland 86% of sporadic coeliac disease cases were found to be DQ2-positive (Polvi et al. 1998). The data available on DQ2-negative coeliac disease patients indicate that they almost invariably express either the DR4 DQ8 (DQA1*03+DQB1*0302) haplotype, or either the DQA1*0501 or the DQB1*02 part of the DQ2 heterodimer (Spurkland et al. 1992, Fernandez-Arguero et al. 1995, Michalski et al. 1996, Polvi et al. 1998).

Previous family studies of coeliac disease have set on with the assumption that there is at least one important non-HLA locus contributing to the development of the disease (Pena et al. 1978, Risch 1987). It has in fact been suggested that this locus might be a stronger determinant of inheritance of coeliac disease than the HLA locus (Risch 1987). For the present, results of recent genome-wide screening studies have been somewhat conflicting, providing evidence for several candidate non-HLA gene regions linked to coeliac disease (Zhong et al. 1996, Houlston et al. 1997, Greco et al. 1998).

Pathogenesis

The jejunal mucosal lesion characteristic of coeliac disease reveals markedly increased cellular and humoral immunity, affording supporting evidence for the subsequent theory that gluten-sensitive enteropathy is immune-mediated. Ingested gluten is the major

environmental trigger, but exposure to adenovirus has also been held to play some part (Kagnoff et al. 1984, Lähdeaho et al. 1993).

Cell-mediated abnormalities in coeliac disease include infiltration of lymphocytes into the epithelium and lamina propria even before morphological changes are evident in the small intestine (Marsh 1992). The increased intraepithelial lymphocyte population consists mainly of cluster design (CD)3+CD8+TCRαβ-positive lymphocytes (Brandtzaeg et al. 1989). Several studies have also demonstrated a significant increase in the numbers of TCRγδ+ intraepithelial lymphocytes in coeliac mucosa (Halstensen et al. 1989, Spencer at al. 1989, Savilahti et al. 1990). The role of intraepithelial lymphocytes in the pathogenesis of coeliac disease is still obscure. There are data indicating that in untreated coeliac disease CD8+TCRαβ+ cells may be activated cytotoxic T-cells, since their increase parallels an increase in the number of intraepithelial lymphocytes containing granzyme B, a protein characteristic of activated cytotoxic lymphocytes (Oberhuber et al. 1996). A recent observation that mice lacking TCRγδ+ lymphocytes developed much more severe intestinal lesions than normal mice during intestinal infection by Eimeria vermiformis suggests that TCRγδ+ T-cells have a protective role against epithelial lesions (Roberts et al. 1996). TCRγδ+ T-cells are known to express keratinocyte growth factor, which may be important for epithelial cell growth or repair of damaged epithelial cells (Boismenu and Havran 1994).

Experimental studies have demonstrated that activation of lamina propria T-cells *in vitro* produces an increase in crypt cell proliferation and villous atrophy (MacDonald and Spencer 1988). There are observations that gliadin-specific CD4+TCRαβ+ T-cells are commonly found in coeliac mucosa and that these cells are activated by gliadin peptides presented mainly by the disease-associated HLA-DQ2 molecules (Lundin et al. 1993, Molberg et al. 1997). Enhanced cytokine production of such activated T-cells may

contribute to mucosal damage (Trejdosiewics and Howdle 1995). It has also been suggested that T-cell activation in the lamina propria results in increased production of matrix metalloproteinases, which by degrading the lamina propria matrix represent a major pathway by which T-cells cause injury in the gut (Pender et al. 1997). It is interesting in this context that gluten-specific T-cells can also be found in the peripheral blood (Gjertsen et al. 1994).

A characteristic of the coeliac disease lesion is also an accumulation of plasma cells producing IgA, IgG and IgM (Savilahti 1972). Further, it has been demonstrated that *in vitro* culture of biopsies produces antibodies to gliadin (Falchuk and Strober 1974), and high titres of IgA- and IgG-class AGA are found in the sera of coeliac disease patients (Table 1, p. 24). It has been suggested that AGA damage the mucosa by way of an antibody-dependent cell-mediated cytotoxic reaction (Levenson et al. 1985). The mucosal lesion has also been claimed to be mediated via complement activation, as a correlation has been shown between the gliadin IgG antibody level and the activation of subepithelial complement (Halstensen et al. 1992).

Gluten-induced IgA-class serum EmA and ARA, which appear to be highly exclusive to patients with untreated coeliac disease (Mäki 1995), are also originally synthesized at intestinal level (Mawhinney and Lowe 1975, Picarelli et al. 1996). The antigen recognised by EmA is now known to be tTG (Dieterich et al. 1997). The expression of tTG is increased in untreated coeliac disease, and gliadin, which has been shown to bind to tissues along reticulin fibres (Unsworth et al. 1987), is the preferred substrate of this enzyme (Bruce et al. 1985). tTG might be important in the formation of gliadin-gliadin or gliadin-tTG complexes, giving rise to novel antigenic epitopes, which could initiate an immune response finally directed against tTG and gliadin (Dieterich et al. 1997). Alternatively, Sollid et al. (1997) have proposed that when such complexes exist,

gliadin-specific T-cells could promote the production of coeliac disease-specific autoantibodies by tTG-reactive B-cells. Recent reports reveal that the deamidation of gliadin peptides by tTG results in better binding of gliadin peptides to HLA DQ2/DQ8 and enhanced peptide recognition by gliadin-specific gut-derived T cells from coeliac disease patients (Molberg et al. 1998, van de Wal et al. 1998). Further, it has been suggested that generation of epitopes by enzymatic modification may be relevant to the breaking of tolerance and the initiation of autoimmune disease (Molberg et al. 1998).

The coeliac disease-specific autoantibodies, EmA and ARA (i.e. tTG antibodies), might also be implicated in the pathogenesis of coeliac disease. tTG is necessary for proteolytic activation of latent transforming growth factor β (Nunes et al. 1997). This activated factor, in turn, is needed for epithelial differentiation (Kurokawa et al. 1987, Halttunen et al. 1996). Recently, Halttunen and Mäki (1999) have shown that coeliac disease serum IgA as well as tTG antibodies disturb transforming growth factor β-mediated fibroblast-epithelial cell cross-talk in an *in vitro* crypt-villus axis model, resulting in inhibition of epithelial cell differentiation and increased proliferation. They suggest that the EmA/ARA, specific for tTG, might also disturb the biological function of transforming growth factor β *in vivo*. Further research, however, is needed to establish whether and how tTG antibodies contribute to the formation of the jejunal mucosal lesion in coeliac disease.

Antibodies in coeliac disease

Gliadin antibodies

Serum AGA represent antibodies to the cereal protein. These antibodies have been detected by various methods, some of which (e.g. immunofluorescence techniques,

solid-phase radioimmunoassay) have shown sufficient sensitivity and precision. In routine clinical practice, ELISA methods for measurement of isotype-specific AGA seem to be most widely accepted (Troncone and Ferguson 1991).

Serum AGA, which are predominantly of the IgA and IgG classes, have frequently been found in untreated coeliac disease (Kilander et al. 1983, Savilahti et al. 1983, Ståhlberg et al. 1986, Ascher et al. 1990, McMillan et al. 1991, Ferreira et al. 1992, Bodé and Gudmand-Hoyer 1994, Lerner et al. 1994, Sategna-Guidetti et al. 1995, Vogelsang et al. 1995, Bottaro et al. 1997). However, results of the numerous studies assessing the diagnostic value of AGA in coeliac disease have been particularly heterogeneous, even with the use of the same basic methodology. Sensitivity for IgA-class AGA has ranged between 31% to 100% and 46% to 100% for IgG-class antibodies (Table 1). The specificity reported for IgA-class antibodies ranges between 68% and 100%, and for IgG-class between 36% and 100%.

Controversy exists regarding the value of specific class of AGA in the diagnosis of coeliac disease. Some investigators recommend the IgA-class AGA test (Savilahti et al. 1983), while others favour the IgG-class AGA test (Bürgin-Wolff et al. 1983, Kelly et al. 1987). In general, AGA tests have correlated satisfactorily with mucosal atrophy in young children (Savilahti et al. 1983, Ascher et al. 1990, Bürgin-Wolff et al. 1991), whereas in adults the association between AGA and coeliac disease is not so marked (O'Farrelly et al. 1983, Grodzinsky et al. 1990, Volta et al. 1990, Mäki et al. 1991c).

Table 1. Sensitivity and specificity of serum gliadin antibodies in untreated coeliac disease.

Authors	Number of		IgA-class		IgG-class	
	patients	controls	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Studies in children						
Ascher et al. (1990)	36	92	97	92		
Bottaro et al. (1997)	50	25	92	68	50	36
Kilander et al. (1983)	8	8	100	100	100	100
Lerner et al. (1994)	34	41	52	94	88	92
Ståhlberg et al. (1986)	31	278	90	86	94	67
Studies in adults						
Bodé & Gudmand-Hoyer (1994)	13	87	46	98	62	97
Ferreira et al. (1992)	21	160	90	85	76	88
Kilander et al. (1983)	36	54	67	94	78	94
Mäki et al. (1991c)	13	109	31	87	46	89
McMillan et al. (1991)	28	68	100	100	57	87
Sategna-Guidetti et al. (1995)	100	109	55	100	78	82
Vogelsang et al. (1995)	49	53	82	83	73	74

Unfortunately, AGA can be found in non-coeliac individuals (Grodzinsky et al. 1992, Pettersson et al. 1993) as well as in patients with other disorders, including Crohn's disease, and other gastrointestinal diseases (Unsworth et al. 1983, Scott and Brandtzaeg 1989). Moreover, at least in certain geographical locations, AGA increase with age in healthy individuals (Uibo et al. 1993). Mäki et al. (1991c) showed that first-degree relatives of coeliac disease patients having normal jejunal mucosa but positive for AGA were genetically different from the probands. Iltanen and collegues (1999b) also reported that in children clinically suspected of coeliac disease and eating normal amounts of gluten, AGA do not correlate with HLA-DQ2. However, some false AGA-positive healthy subjects may in fact be undiagnosed coeliacs. Kaukinen et al. (1998)

showed that AGA-positive adult patients with normal small-bowel mucosal morphology frequently bear the immunohistochemical markers of coeliac disease latency.

Since AGA titres disappear after the introduction of a gluten-free diet (Savilahti et al. 1983, Bürgin-Wolff et al. 1991), their presence or absence has been claimed to be an indicator of dietary compliance. After gluten is reintroduced into the diet most coeliac disease patients produce AGA (Mayer et al. 1989). However, Bürgin-Wolff et al. (1991) showed that after prolonged periods on gluten-containing food, AGA titres in some individuals decreased again and even became negative despite gluten and a pathological mucosa.

Reticulin, endomysial and jejunal autoantibodies

In 1971, Seah et al. originally detected ARA in the sera of adult coeliac disease and dermatitis herpetiformis patients. They were described as staining of connective tissue fibres around hepatic sinusoids and blood vessels as well as perilobular, periglomerular, and occasionally glomerular staining of the kidney, and also fine staining of the stroma between gastric glands on the stomach mucosa. In connective tissue ARA appears to be directed against silver-stain-positive ‘reticulin’ fibres. The antibody is best detected by the indirect immunofluorescence method using unfixed cryostat sections of rat kidney and liver as antigens. Rizzetto and Doniah (1973) described several types of immunofluorescence patterns of ARA; R_1, R_2, R_S, K_C and AC. Of these, only the R_1-type staining pattern has been exhibited by coeliac disease and dermatitis herpetiformis patients. This is characterised by staining of peritubular and periglomerular fibres in the kidney and fluorescence in portal tracts of the liver (Magalhaes et al. 1974, Eade et al. 1977) closely resembling the fibrillar staining pattern produced in tissues by silver-impregnation staining (Kárpáti et al. 1991).

Serum ARA are of both IgA and IgG class, of which the IgA-class ARA seems to be a highly sensitive and disease-specific marker of coeliac disease (Seah et al. 1971, Mäki et al. 1984a). Figures for sensitivity range between 44% and 100% and for specificity between 86% and 100% (Table 2).

Table 2. Sensitivity and specificity of serum IgA-class reticulin and endomysial antibodies in untreated coeliac disease.

Authors	Number of		Reticulin antibodies		Endomysial antibodies	
	patients	controls	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Studies in children						
Bottaro et al. (1997)	50	25	74	100	96	96
Hällström (1989)	14	24	100	100	100	100
Kolho and Savilahti (1997)	53	114	96	92	94	100
Lerner et al. (1994)	34	41	65	100	97	98
Mäki et al. (1984a)	29	245	97	98		
Sacchetti et al. (1996)	32	42	94	100	97	100
Seah et al. (1971)	10	28	90	100		
Volta et al. (1991)	29	20	52	100	90	100
Studies in adults						
Ferreira et al. (1992)	21	160	90	99	100	99
Hällström (1989)	35	145	91	100	91	100
Mäki et al. (1991c)	13	109	92	95	92	95
Sategna-Guidetti et al. (1997)	104	94			95	100
Valdimarsson et al. (1996)	19	125			74	100
Volta et al. (1991)	41	20	44	100	85	100

IgA-class ARA-positivity clearly predicts clinically silent coeliac disease among close relatives of coeliac patients (Mäki et al. 1991c), and patients with type I diabetes (Mäki et al. 1984b, Collin et al. 1989, Rossi et al. 1993, Acerini et al. 1998), Sjögren's

syndrome (Collin et al. 1992b) and autoimmune thyroid disease (Collin et al. 1994b). In addition, ARA are occasionally discovered in the course of routine autoantibody testing. Patients in whom IgA-class ARA are found fortuitously most often have coeliac disease irrespective of clinical conditions (Unsworth and Brown 1994). IgG-class ARA seldom occur alone without IgA-class ARA, but the specificity of IgG-class ARA for coeliac disease is controversial (Kumar et al. 1989). On the other hand, the association between coeliac disease and selective IgA deficiency results in negative results on IgA-class ARA and may make for underdiagnosis; thus, patients with IgA deficiency should also be assessed for serum IgG-class ARA or AGA (Collin et al. 1992a).

In 1983, Chorzelski et al. described a new IgA-class tissue antibody directed against the 'reticulin-like' silver-stain-positive intermyofibrillar substance of the monkey oesophagus smooth muscle, termed endomysium, and this finding was strongly associated with the presence of coeliac disease. The IgA-class EmA test has since gained acceptance as a sensitive and specific method of identifying coeliac subjects (Hällström 1989, Volta et al. 1991, Mäki et al. 1991c, Ferreira et al. 1992). The reported sensitivity for IgA-class EmA in patients with untreated coeliac disease ranges between 74% and 100% (Table 2). Specificity for IgA-class EmA has ranged between 95% to 100%. The occurrence of EmA has been found to be age-dependent, the antibody being reported notably less frequently in coeliac disease patients younger than two years compared with older patients (Bürgin-Wolff et al. 1991).

Coeliac disease patient sera have been reported also to react with human tissues. Seah et al. (1971) found a clear ARA fluorescence when they used human foetal liver, small intestine and skin as substrate. Hällström (1989) showed a strong fluorescence in human adult and foetal liver, spleen, lung, jejunum, thymus and pancreas, and a weak reaction in human adult kidney and skin and foetal colon with ARA positive sera. IgA-class

jejunal antibodies binding to normal jejunum from healthy children and showing similarities to both ARA and EmA have been described in the sera of dermatitis herpetiformis patients (Kárpáti et al. 1986) and in coeliac disease patients (Kárpáti et al. 1990). Recently, Ladinser et al. (1994) detected ultrastructural binding sites of EmA on human umbilical cord. It has been suggested that human umbilical cord could provide an inexpensive and sensitive substitute for the oesophagus of endangered species (Table 3).

Table 3. Sensitivity and specificity of serum IgA-class endomysial antibodies on monkey oesophagus and human umbilical cord in untreated coeliac disease.

Authors	Number of		IgA-class EmA			
			monkey oesophagus		human umbilical cord	
	patients	controls	Sensitivity (%)	Specificity (%)	Sensitivity (%)	Specificity (%)
Studies in children						
Bottaro et al. (1997)	50	25	96	96	94	100
Kolho and Savilahti (1997)	53	114	94	100	94	100
Sacchetti et al. (1996)	32	42	97	100	93	100
Studies in adults						
Sategna-Guidetti et al. (1997)	104	94	95	100	95	91
Volta et al. (1995)	60	100	95	100	95	100

Gluten-induced coeliac disease-specific ARA and EmA mostly disappear within one year on a gluten-free diet (Mäki et al. 1984a, Hällström 1989). These tests also seem to be suitable for the prediction of mucosal relapse in coeliac disease (Mäki et al. 1989, Bürgin-Wolff et al. 1991). Moreover, positive ARA or EmA in individuals with normal small-bowel mucosa evidently reveals latent coeliac disease (Mäki et al. 1990, Collin et

al. 1993, Iltanen et al. 1999b). Collin et al. (1993) reported that 83% ARA-positive patients with normal villous architecture developed coeliac disease during follow-up. It has also been shown that ARA positivity is associated with coeliac-type HLA DQA1*0501 and DQB1*0201 alleles (Mäki et al. 1991c, Iltanen et al. 1999b).

There seem to be some species-specific differences among the reticulin antigens (Hällström, 1989, Valeski et al. 1990, Yiannakou et al. 1997), and it has also been surmised that there might be even intraspecies differences (Valeski et al. 1990). Hällström (1989) showed that the rodent–specific IgA- and IgG-class antibodies could be absorped from patient sera with rodent (rat, mouse, guinea pig) liver homogenates, while a strong positivity against human tissues and monkey oesophagus persisted. In contrast, after absorption with human liver homogenates both rat and human tissues and monkey oesophagus all gave negative results. Absorption studies by Kárpáti et al. (1990) suggest that EmA and jejunal antibodies are one and the same; both were absorbed by crude reticulin preparations of human or monkey origin.

The antigen recognised by ARA, EmA and jejunal antibodies is highly expressed in rodent and primate, including human, tissues (Chorzelski et al. 1983, Hällström 1989, Kárpáti et al. 1990), and these tissue antibodies have been taken to be the target organ-related IgA-class autoantibodies implicated in coeliac disease (Kárpáti et al. 1990). Coeliac disease patient serum IgA-class antibodies have been found to recognise an amorphous component adjacent to collagenous–reticulin fibrils in the connective tissue (Kárpáti et al. 1992). It has been postulated that ARA and EmA are autoantibodies generated against human reticulin (Mäki et al. 1991a). Immunohistological studies have shown that type III collagen, noncollagenous reticulin component and fibronectin are co-distributed in tissues in a manner consistent with their being components of reticulin (Unsworth et al. 1982). However, it has been shown that ARA-positive patient sera do

not react with these tissue components (Unsworth et al. 1984). Mäki et al. (1991a) demonstrated that IgA-class ARA and EmA from coeliac patients sera react with extracellular matrix non-collagenous molecules in the human foetal lung. The autoantigen has been shown to be expressed by human fibroblasts (Marttinen and Mäki 1993). Further, 28- 62- and 66-kD proteins reactive with coeliac disease-specific antibodies have been isolated from rat enterocyte surface membrane (Tuckova et al. 1995). Whelan et al. (1996) showed that an antigen found in human umbilical vein endothelial cells is antigenically similar to that found in reticulin and endomysium.

Tissue transglutaminase autoantibodies

Only recently, Dieterich et al. (1997) immunoprecipitated with untreated coeliac sera a single protein band from lysed human fibrosarcoma cells. Cleavage products of this 85-kD autoantigen were subjected to aminoterminal sequence analysis. Sequences were assigned to tTG (EC 2.3.2.13), which belongs to a diverse family of calcium-dependent enzymes widely distributed among the tissues and body fluids of vertebrates. These enzymes catalyse the covalent cross-linking of specific proteins by the formation of isopeptide bonds between γ-carboxyl groups of glutamine residues in one polypeptide and ε-amino groups of lysine residues in another. tTG is mainly regarded as an intracellular enzyme, but it has been shown to be released during wounding (Upchurch et al. 1991). It is capable of cross-linking fibronectin, collagen II, V, XI and procollagen II, and it may have a role in stabilising the extracellular matrix. In active coeliac disease the expression of tTG is increased and the enzyme accepts gliadin as one of its substrates (Bruce et al. 1985). Dieterich et al. (1997) demonstrated that the pretreatment of coeliac disease patient serum with tTG almost completely abolishes EmA staining and concluded that they had identified tTG as the unknown endomysial autoantigen.

Further, using commercially available guinea pig tTG as a substrate in an ELISA test, they showed that 12 coeliac patients but none of the seven controls displayed increased IgA immunoreactivity. Thus, tTG-based ELISA might represent a potential tool for the diagnosis and follow-up of the disease (Dieterich et al. 1997).

Purpose of the present study

The aims of the present study were:

1. to assess the value of human umbilical cord and Wharton's jelly-derived fibroblasts as antigens in screening tests for coeliac disease (I, II);

2. to establish whether IgA-class tissue transglutaminase autoantibodies can be considered specific for coeliac disease (III);

3. to find out whether the tissue distribution of transglutaminase is compatible with the coeliac disease-specific reticulin, endomysial and jejunal autoantibody staining patterns (IV).

Materials and methods

Study groups

Coeliac disease patients

Serum samples were obtained from 276 patients with untreated coeliac disease examined at the Departments of Paediatrics or Medicine at Tampere University Hospital or at the Children's Hospital, Helsinki University (Table 4). Of these 276 patients, 173 were paediatric patients (median age 7.5 years, range 0.8-17.3) (**I, III**) with untreated coeliac disease. All exhibited a flat mucosa or severe villous atrophy on initial small-bowel mucosa and fulfilled the criteria of ESPGAN (Mäki et al. 1989, Walker-Smith et al. 1990). In addition, study was made of serum samples available from paediatric patients on a gluten-free diet for at least one year (**I**; n=62, **III**; n=38), during gluten challenge at the time of mucosal relapse (**I**; n=16, **III**; n=18), and again after the reintroduction of a gluten-free diet (**I**; n=16, **III**; n=11).

In 92 adult patients with untreated coeliac disease (median age 43.2 years, range 20.2-72.3) subtotal or severe partial small-bowel villous atrophy with crypt hyperplasia was considered the diagnostic criterion for coeliac disease (**II)**. Mucosal healing on gluten-free diet confirmed the diagnosis in all patients. Twenty-nine of these 92 patients, together with a further eleven adults with untreated coeliac disease were recruited in study **III**.

In addition, sera from 14 untreated coeliac disease patients with selective IgA deficiency (serum IgA concentration <0.05 g/l) were available for study **III**, four of them also having been included in study **II**.

Table 4. Distribution of coeliac disease patients and controls in studies **I-III.**

Subjects	Study **I**		Study **II**		Study **III**	
	Number of patients	Median age (range)	Number of patients	Median age (range)	Number of patients (participating also in study **I** or **II**)	Median age (range)
Patients with untreated coeliac disease						
Children	88	7.5 (0.8-16.6)			96 (11)	6.7 (0.8-17.3)
Adults			92	43.2 (20.2-72.3)	40 (29)	45.3 (24.5-69.3)
IgA-deficient subjects			4		14 (4)	
Coeliac disease patients						
on a gluten-free diet	62				38 (19)	
challenged with gluten	16				18 (11)	
Control children*						
clinically suspected of coeliac disease	110	7.7 (0.5-17.1)			84 (28)	6.2 (0.8-14.3)
recurrent abdominal pain	30	10.5 (2.8-15.4)			26 (26)	10.5 (2.8-15.4)
Crohn's disease	6	13.0 (8.5-15.8)			16 (5)	15.4 (5.8-19.5)
ulcerative colitis	6	13.0 (5.3-14.5)			16 (5)	13.4 (5.3-16.8)
type I diabetes					21	11.9 (6.3-19.0)
Control adults*						
Dyspepsia			52	44.3 (16.3-76.0)	44 (44)	43.5 (16.3-76.0)
Crohn's disease			14	31.3 (26.5-81.4)		
ulcerative colitis			29	31.5 (19.9-70.3)		

*All disease controls evinced normal villous architecture in small-bowel specimens.

Control patients

The study population included altogether 346 controls. Of these, 251 were children (median age 9.0 years, range 0.5-19.5) examined at the Department of Paediatrics at Tampere University Hospital or at the Children's Hospital, Helsinki University (Table 4). Sera were drawn from 166 children (median age 6.9 years, range 0.5-17.1) with a suspicion of coeliac disease, but found to have normal small-bowel villous architecture, and from 30 children (median age 10.5 years, range 2.8-15.4) who had undergone gastroscopy for recurrent abdominal pain and in whom a diagnosis of coeliac disease was excluded on distal duodenal biopsy. Other control patients had Crohn's disease (n=17; median age 14.9 years, range 5.8-19.5), ulcerative colitis (n=17; median age 13.3 years, range 5.3-16.8) or type I diabetes (n=21; median age 11.9, range 6.3-19.0).

Fifty-two of the 346 controls were adults (median age 44.3 years, range 16.3-76.0) attending the Department of Medicine at Tampere University Hospital for gastroscopic examination because of dyspepsia (Table 4). In addition, 14 patients with Crohn's disease (median age 31.3 years, range 26.5-81.4) and 29 with ulcerative colitis (median age 31.5 years, range 19.9-70.3) found on small-bowel biopsy to have normal villous architecture were included in study **II**. One control patient had selective IgA deficiency (**II**).

Patient sera and gliadin antibodies in study IV

Six IgA-class ARA- and EmA-positive untreated coeliac disease patient sera, four IgA-class ARA- and EmA-negative non-coeliac control sera and an IgG-class EmA-positive serum sample from an IgA-deficient untreated coeliac disease patient were used in study **IV**.

Gluten-free BALB/c mice were immunised with tTG (T5398, lot 37H9564; Sigma Chemical Co., St. Louis, USA), tTG complexed with gliadin or gliadin (Sigma G3375) according to the procedure described in study **IV**.

Serum antibody tests

Endomysial and reticulin autoantibodies

IgA-class EmA and R_1-type ARA were determined in all 276 patients with untreated coeliac disease, in 346 control subjects and in 14 coeliac patients with IgA deficiency by indirect immunofluorescence using unfixed cryostat sections of full-term human infant umbilical cord for EmA (Ladinser et al. 1994) and a composite block of rat kidney, liver, stomach and heart for ARA (Mäki et al. 1984a, Hällström 1989) as antigens (**I-III**). Human umbilical cord sections were prepared as described in study **I.** The Gomori method of silver-staining was used to detect the presence of reticulin fibrils in cord sections. IgA-class EmA were also detected in a subgroup of 38 children with untreated coeliac disease and 42 controls on commercial slides of monkey oesophagus (Viro-Immun Labor-Diagnostica, Oberursel, Germany) (Chorzelski et al. 1983, Hällström 1989) for comparative purposes and will be referred to here as monkey oesophagus EmA (**I**). In addition, IgG-class EmA and ARA were determined in 92 adults with untreated coeliac disease, in 95 controls (**II**) and in 14 IgA-deficient subjects with untreated coeliac disease (**II-III**). For IgG-class EmA both full-term and preterm human infant (gestation age 24 weeks) umbilical cord sections were used as substrate.

All tissue sections were processed in the same way. Slides were incubated for 30 min with serum samples diluted 1:5 and 1:50 in phosphate-buffered saline (PBS). After washing thrice in PBS, the sections were incubated with fluorescein isothiocyanate (FITC)-conjugated monospecific goat antiserum to human IgA or IgG (Sanofi

Diagnostics Pasteur, Inc., Chaska, USA). After washing and after addition of mounting medium, sections were read by fluorescence microscopy blindly by two members of the group. A serum dilution of 1:≥5 was considered positive. Positive samples were further diluted up to 1:8000. Fluorescence was compared with positive and negative controls tested in each assay.

Tissue transglutaminase autoantibodies

In our pilot study microtitre plates (Nunc Immuno Plate Maxisorb; Nunc A/S) were coated with 1 μg tTG from guinea pig liver (Sigma T5398) in 100 μl PBS (pH 7.4) as previously described (Dieterich et al. 1997) **(III)**. Serum samples from 20 untreated coeliac disease patients and from 24 controls were examined.

In the main study serum samples from 136 patients with untreated coeliac disease, from 207 controls and from 14 coeliac patients with IgA deficiency (Table 4) were examined for IgA- and IgG-class tTG autoantibodies with ELISA using microtitre plates coated with 1 μg tTG (Sigma T5398) in 100 μl 0.05 M Tris-buffered saline (TBS) with 5 mM $CaCl_2$, pH 7.5 **(III, IV)**. Between the steps the plates were washed with 0.05 M TBS with 0.01 M EDTA, and 0.1% Tween-20 (TTBS). Serum samples diluted 1:100 in 0.05 M TTBS were added to the wells as duplicates and then incubated for one hour at room temperature. After that, the plates were incubated with peroxidase-conjugated mouse antiserum to human IgA or IgG (Dako A/S, Glostrup, Denmark) diluted 1:2000 in the same buffer. Colour was developed by the addition of 100 μl 0.1 M sodium citrate, 1 mg/ml o-phenylenediamine dihydrochloride (Dako), 0.06% H_2O_2, pH 4.2, at room temperature for 30 min. Absorbances were read spectrophotometrically at 450 nm. Positive and negative control serum samples, positive reference serum provided by the European Medical Research Councils Clinical Network for Gastroenterological

Immunology and the ESPGAN Working Group for Serological Screening of Celiac Disease (Stern et al. 1997), and buffer blanks were included in each assay. The antibody concentrations were expressed in arbitrary units (AU), i.e. as percentages of the positive reference serum.

Human umbilical cord-derived whole-cell fibroblast autoantibodies

For whole-cell ELISA Wharton's jelly-derived fibroblasts isolated as described in study **I** were allowed to attach to ELISA plates (Nunc MicroWell Plate; Nunc A/S) for two hours at 37 ^{o}C in humidified atmosphere. After each step the plates were washed with PBS. The cells were fixed with Baker's fixative for 10 min and quickly treated with absolute ethanol. Serum samples from 27 randomly selected children with untreated coeliac disease and 66 control children, diluted 1:100 with PBS containing 1% bovine serum albumin and 0.1% Tween 20 (pH 7.4) were incubated and screened with peroxidase-conjugated mouse antiserum to human IgA (Dako; dilution 1:5000). O-phenylenediamine dihydrochloride (Dako) dissolved in 0.1 M citric acid-phosphate buffer (pH 5.0) was used as substrate for peroxidase. The colour intensity was measured spectrophotometrically at 492 nm. Four replicates were used for each serum sample, and positive and negative control serum pools and buffer blanks were included in each assay.

The following equation was used to calculate the results: $AU = \frac{S_{od} - Np_{od}}{100 - Np_{od}}$, AU referring to arbitrary units, S_{od} to sample optical density under analysis, and Np_{od} to the optical density of the negative control serum pool. The optical density of the positive control serum pool was given the value 100 AU.

Gliadin antibodies

The whole study population was analysed for IgA- and IgG-class AGA in according to standard ELISA with a crude gliadin (Sigma G3375) as antigen (Savilahti et al. 1983).

Western blot analysis and blocking studies

Western blot analysis of tTG with coeliac disease sera, control sera and mouse monoclonal antibody against tTG (mAb-tTG CUB7402; NeoMarkers, Fremont, USA) was performed under nonreducing and reducing conditions as described in study **III**.

In tTG antibody ELISA blocking experiment coeliac disease sera were preincubated with 0.05-150 μg tTG (Sigma T5398) in PBS or in 0.05 M TBS with 5 mM $CaCl_2$ before being tested by ELISA (**III**).

Immunofluorescent staining studies

Coeliac and non-coeliac patient sera, mAb-tTG (CUB 7402 and TG 100; NeoMarkers) and tTG-immunized mouse sera were analysed by indirect immunofluorescence for antibodies giving coeliac-type tissue staining pattern using a composite block of rat tissues (Mäki et al. 1984a), monkey oesophagus (Chorzelski et al. 1983), human umbilical cord (Ladinser et al. 1994) and biopsy samples from normal human jejunum (Kárpáti et al. 1990) as substrates. Further, Wharton's jelly-derived fibroblasts were isolated and used as substrate as described in study **I**. FITC-conjugated goat antibodies against human IgA or IgG (Sanofi Diagnostics Pasteur; dilution 1:120) and rabbit antibodies against mouse immunoglobulin subclasses (Dako; dilution 1:100) were used as secondary antibodies. Tissue localisation of fibronectin was also studied on the same substrates with polyclonal rabbit antiserum to human fibronectin (Dako; dilution 1:300)

as primary antibody and FITC-conjugated swine anti-rabbit immunoglobulins (Dako; dilution 1:100) as secondary antibody.

Double immunofluorescent studies were carried out using rodent and primate, including human, tissues described above as substrates. The various primary antibody combinations used are listed in Table 1 in study **IV**. FITC-conjugated goat anti-human IgA (Sanofi Diagnostics Pasteur) and tetra-methyl-rhodamine isothiocyanate isomer R (TRITC)-conjugated rabbit anti-mouse IgG were used as secondary antibodies. Fibronectin antibodies were examined with TRITC-conjugated goat antibodies against rabbit immunoglobulins (Boehringer, Mannheim, Germany). The study protocol is summarised in study **IV**.

The procedure for studying the competition of coeliac patient sera or IgA-deficient coeliac serum samples with mAb-tTG CUB7402 (NeoMarkers) is described in detail in study **IV.**

Removal of tissue transglutaminase from tissue sections and absorption studies

Human umbilical cord and monkey oesophagus sections were pretreated with 2 M potassium-thiocyanate (KSCN) (Sigma) before being stained for EmA **(IV)**. In order to rebind tTG, KSCN-treated sections were incubated with tTG (Sigma T5398) prior to the immunofluorescent stainings.

The binding of EmA, ARA and AGA to coeliac disease-specific autoantigen protein molecules (Marttinen and Mäki 1993) (**I**) or to tTG (**IV**) was evaluated according to the affinity chromatography experiments described in the original papers.

Statistical analysis

Statistical analysis was based on 95% confidence intervals. Computation was carried out using CIA software (Gardner and Altman 1989). The sensitivity was defined as the frequency of positive antibody results in patients with coeliac disease, and the specificity as the frequency of negative antibody results in patients excluded for coeliac disease.

Ethics

The study protocols were approved by the Ethical Committee of Tampere University Hospital (I-IV). Permission to use the umbilical cord as substrate for EmA testing was asked from the mother.

Results

Endomysial and reticulin autoantibodies

Description of endomysial antibodies on human umbilical cord

On cryostat sections of human umbilical cord immunofluorescence staining positivity was seen in the vessel wall smooth muscle endomysium as a honeycomb reticular network and also in the cytoplasm of the Wharton's jelly fibroblasts (**I**; Figure 1). The pattern was identical to that observed with silver staining of the cord sections.

The interpretation of the serum IgG-class EmA test using full-term infant umbilical cord as antigen was interfered with the intense background staining. The umbilical cord of preterm infants (gestation age 24 weeks) brought out the specific honeycomb-like EmA staining pattern (**II**).

Paediatric patients (I, III)

IgA-class EmA were detected in 170 (98%) and ARA in 171 (99%) of the 173 children with untreated coeliac disease (Table 5). All 38 untreated coeliacs tested for comparative purposes for IgA-class monkey oesophagus EmA were positive (sensitivity 100%).

Only three out of the 251 control children having normal jejunal mucosa were positive for IgA-class EmA, the test yielding in a specificity of 99%. Positive IgA-class ARA titres were found in 20 of the 251 control children (specificity 92%), and EmA on monkey oesophagus in seven of the 42 controls tested (specificity 83%)

Table 5. Frequency of positive endomysial (EmA), reticulin (ARA), tissue transglutaminase (tTG-ab), human umbilical cord-derived whole-cell fibroblast (HUC-Fb-ab) and gliadin antibodies (AGA) in patients with untreated coeliac disease (CD) and in control patients (**I-III**).

	EmA		ARA		tTG-ab	HUC-Fb-ab	AGA	
	IgA (%)	IgG (%)	IgA (%)	IgG (%)	IgA (%)	IgA (%)	IgA (%)	IgG (%)
Patients with untreated CD, all	258/276 (93)	11/92 (12)	253/276 (92)	12/92 (13)	129/136 (95)	27/27 (100)	229/276 (83)	159/276 (58)
Children	170/173 (98)	Not tested	171/173 (99)	Not tested	91/96 (95)	27/27 (100)	147/173 (85)	124/173 (72)
Adults	88/103 (85)	11/92 (12)	82/103 (80)	12/92 (13)	38/40 (95)	Not tested	82/103 (80)	35/103 (34)
IgA-deficient subjects with CD	0/14	14/14 (100)	0/14	14/14 (100)	0/14	Not tested	0/14	5/8 (63)
Controls, all*	3/346 (1)	0/95	20/346 (6)	2/95 (2)	13/207 (6)	12/66 (18)	80/346 (23)	93/346 (27)
Children, all	3/251 (1)	Not tested	20/251 (8)	Not tested	4/163 (2)	12/66 (18)	67/251 (27)	90/251 (36)
clinically suspected of CD	3/166 (2)	Not tested	12/166 (7)	Not tested	3/84 (4)	5/34 (12)	56/166 (34)	78/166 (47)
recurrent abdominal pain	0/30	Not tested	1/30 (3)	Not tested	1/26 (4)	2/22 (9)	3/30 (10)	2/30 (7)
IBD[§]	0/34	Not tested	3/34 (9)	Not tested	0/32	5/10 (50)	6/34 (18)	7/34 (21)
type I diabetes	0/21	Not tested	4/21 (19)	Not tested	0/21	Not tested	2/21 (10)	3/21 (14)
Adults, all	0/95	0/95	0/95	2/95 (2)	9/44 (20)	Not tested	13/95 (14)	3/95 (3)
Dyspepsia	0/52	0/52	0/52	2/52 (4)	9/44 (20)	Not tested	4/52 (8)	1/52 (2)
IBD[§]	0/43	0/43	0/43	0/43	Not tested	Not tested	9/43 (21)	2/43 (5)

*All disease controls had normal villous architecture in small-bowel specimens.

[§]IBD=inflammatory bowel disease

Figure 1 shows individual IgA-class EmA results in each paediatric patient group studied. In 53 out of 62 (85%) children with coeliac disease IgA-class EmA titres showed negative seroconversion during gluten-free diet treatment for a year or more (group II). All 16 initially EmA-negative children who were challenged with gluten were positive for antibodies at the time of mucosal deterioration (group III). The antibody titres decreased again when gluten was withdrawn from the diet (group IV).

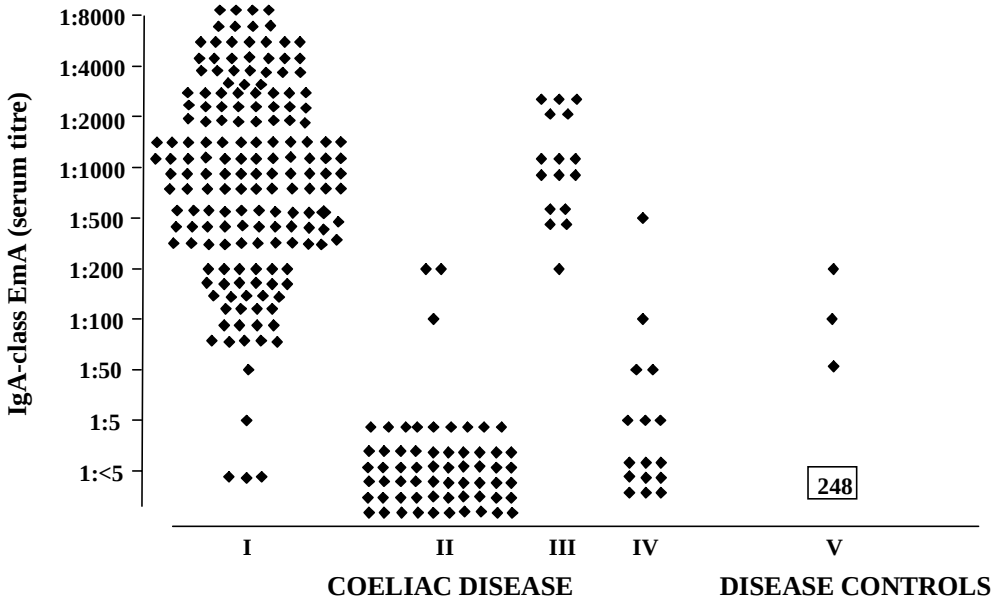

Figure 1. Serum IgA-class endomysial antibody (EmA) titres determined by indirect immunofluorescence method using human umbilical cord as antigen. The following paediatric patient groups were studied: I, newly diagnosed untreated coeliac patients (n=173); II, patients with coeliac disease during gluten-free diet (n=62); III, coeliac patients during gluten challenge (n=16); IV, again after gluten withdrawal (n=16); and V, disease controls with normal small-bowel mucosa (n=251). The *box* in group V represents 248 individuals with negative results.

Adult patients (II, III)

Of the 103 adults with untreated coeliac disease, 88 (85%) had positive IgA-class EmA titres (Table 5). Serum IgA-class ARA were found in 82 (80%) of the patients. Both tissue autoantibody tests attained a specificity of 100%.

Only 11 (12%) of the 92 adults with untreated coeliac disease tested were positive for IgG-class EmA and 12 (13%) for IgG-class ARA, respectively (Table 5). The specificity of the IgG-class EmA test was 100% and that of the IgG-class ARA test 98% (93 out of 95).

All 14 untreated coeliac disease patients with selective IgA deficiency were positive for both IgG-class EmA and ARA.

Tissue transglutaminase autoantibodies (III)

Results of the pilot study with the tTG antibody ELISA using tTG in PBS as substrate (Dieterich et al. 1997) showed that the test did not work in our hands. Immunoblotting and ELISA blocking experiments indicated that calcium is needed for the specific antigen-antibody reaction to occur. The tTG antibody ELISA was thus, performed in the presence of calcium. The cut-off level was set at 10 AU based on the calculations of sensitivity and specificity with different cut-off levels (data not shown). Using this cut-off level a sensitivity of 95% for both children (91 out of 96) and adults (38 out of 40) was achieved. Specificity was 98% (159 out of 163) for children and that for adults 80% (35 out of 44) (Table 5).

Negative seroconversion of tTG antibodies was observed in 23 out of 38 (61%) coeliac disease patients on a gluten-free diet for a year or more. All 18 patients challenged with gluten had elevated tTG antibody levels at the time of mucosal relapse. After gluten withdrawal the antibody titres again decreased.

As demonstrated in Figure 2, the titres of IgG-class tTG antibodies in patients with untreated coeliac disease were no different from those in control subjects. It was thus, not possible to establish the cut-off level for the IgG-class tTG antibody test. However, Figure 2 also shows that IgG-class tTG antibodies over 10 AU (the cut-off level for IgA-class tTG antibody test) were found in all 14 patients with coeliac disease and selective IgA deficiency.

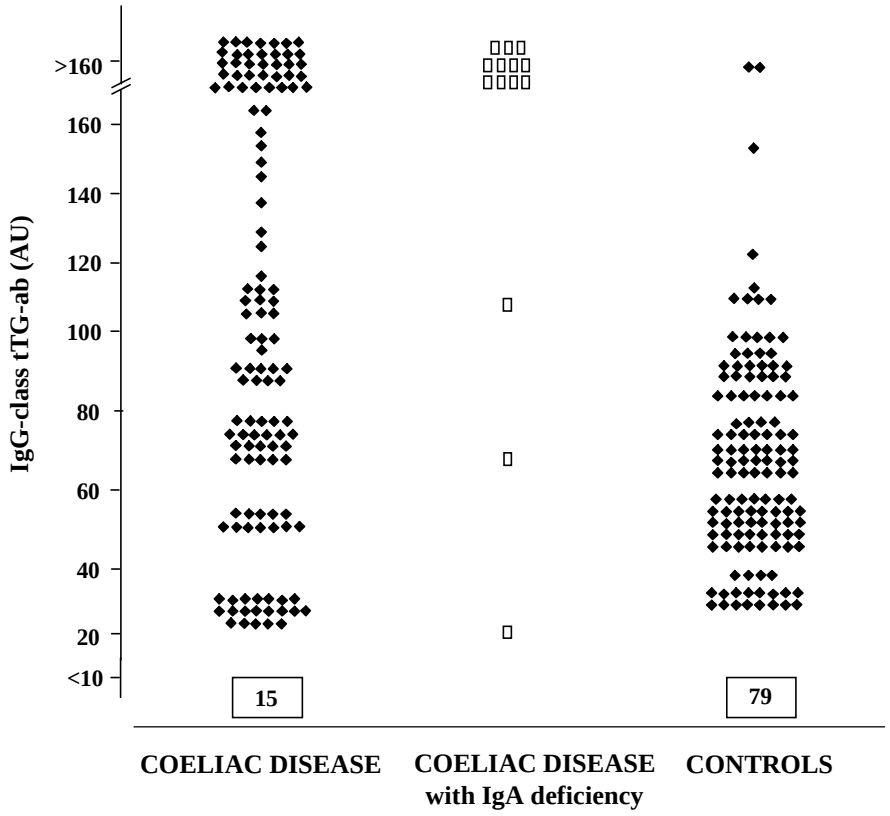

Figure 2. Serum IgG-class tissue transglutaminase antibody (tTG-ab) titres determined by ELISA in 136 patients with untreated coeliac disease (96 children and 40 adults), in 14 coeliac patients with IgA-deficiency and in 207 control patients (163 children and 44 adults). The *boxes* in coeliac patient and control groups represent the number of individuals with negative results.

Human umbilical cord-derived whole-cell fibroblast autoantibodies (I)

The human umbilical cord-derived whole-cell fibroblast autoantibody ELISA test proved to be highly sensitive **(I)**. All 27 untreated coeliac disease children tested had positive titres with a mean AU of 102 (standard deviation 22, range 55-144) with an arbitrary cut-off level of 40 AU for positivity. Twelve out of 66 disease control patients (mean AU 26, standard deviation 28, range 0-131) were positive for these antibodies, the test yielding a specificity of 82%.

Gliadin antibodies (I-III)

IgA-class AGA results showed a sensitivity of 85% (147 of 173) and a specificity of 73% (184 of 251) in detecting untreated coeliac disease in children (Table 5). The sensitivity of the IgG-class AGA test was 72% (124 out of 173), specificity 64% (161 out of 251). Serum IgA-class AGA were found in 82 out of 103 (80%) adult coeliac patients. The specificity of IgA-class AGA test in adults was 86%. The sensitivity of IgG-class AGA test was 34% and the specificity 97%.

Comparison of antibody tests

Figure 3 shows the titres of IgA-class EmA plotted against R_1-type ARA; a good correlation is seen. Thirteen out of 276 untreated coeliac disease patients tested in this study were positive in one or other test only; nine ARA negative patients were positive for EmA, and four EmA-negative were positive for ARA. The IgA-class EmA titres on human umbilical cord correlated also well with EmA titres on monkey oesophagus (**I**; Figure 3B) and with the results of the new whole-cell ELISA test (**I**; Figure 4A). The concordance rate between IgA-class EmA test and the new IgA-class tTG antibody ELISA was 320 (93%) out of 343.

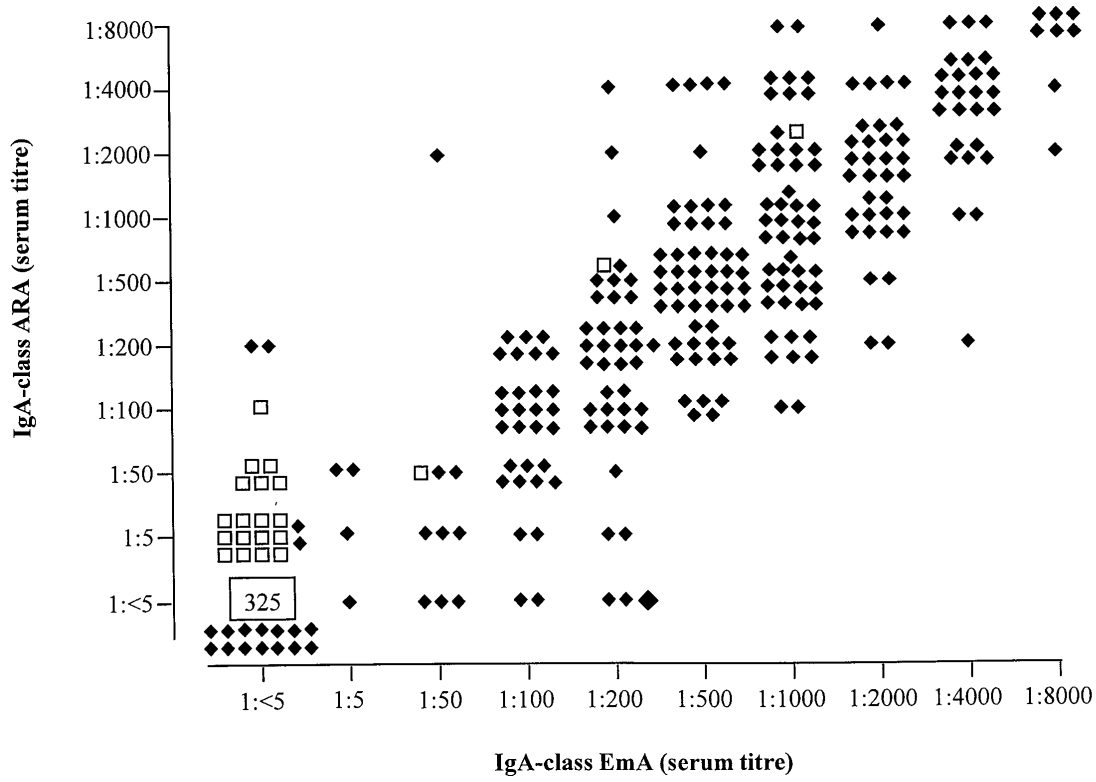

Figure 3. Correlation between serum IgA-class reticulin (ARA) and endomysial (EmA) autoantibodies. ♦, patients with untreated coeliac disease; , control patients with normal jejunal mucosa. The *box* represents 325 disease control patients with negative results in both tests.

The IgA-class AGA test was not as sensitive and specific as other tests used to detect untreated coeliac disease (Table 5). However, in study **II** high IgA-class AGA titres (>0.5 EU/ml) were observed in eight patients with untreated coeliac disease, who were negative for both EmA and ARA.

The 95% confidence interval values calculated for the sensitivity and the specificity of the IgA-class EmA, ARA and tTG antibody tests merged (Table 6). Those for the sensitivity and the specificity of the IgA-class AGA test did not overlap with the values for the IgA-class EmA, ARA and tTG antibody tests in the whole study population. The intervals for the sensitivity of the AGA test, again overlapped with those for tTG

antibody test in children and that for the ARA test in adults. In adults the values for the specificity of the tTG antibody and AGA tests overlapped.

Table 6. 95% confidence intervals (CI) for the sensitivity and the specificity of the IgA-class endomysial (EmA), reticulin (ARA), gliadin (AGA) and tissue transglutaminase (tTG-ab) antibodies in patients with untreated coeliac disease and in controls.

	Antibody test	Sensitivity (95% CI)	Specificity (95%CI)
All	EmA	93.5% (89.9, 96.1)	99.1% (97.5, 99.8)
	ARA	91.7% (87.8, 94.6)	94.2% (91.2, 96.4)
	AGA	83.0% (78.5, 87.4)	76.9% (72.4, 81.3)
	tTG-ab	94.9% (89.7, 97.9)	93.7% (89.5, 96.6)
Children	EmA	98.3% (95.0, 99.6)	98.8% (96.6, 99.7)
	ARA	98.8% (95.9, 99.9)	92.0% (88.0, 95.1)
	AGA	85.0% (79.6, 90.3)	73.3% (67.8, 78.8)
	tTG-ab	94.8% (88.3, 98.3)	97.5% (93.8, 99.3)
Adults	EmA	85.4% (78.6, 92.2)	100% (96.2, 100)
	ARA	79.6 % (71.8, 87.4)	100% (96.2, 100)
	AGA	79.6% (71.8, 87.4)	86.3% (77.7, 92.5)
	tTG-ab	95.0% (83.1, 99.4)	79.5% (64.7, 90.2)

Relationships between endomysial, reticulin and tissue transglutaminase autoantibodies

Immunofluorescent staining studies

In indirect immunofluorescent studies mAb-tTG CUB 7402 and TG 100 bound to all rodent, primate and human tissues studied as well as in cultured fibroblasts derived from Wharton's jelly of human umbilical cord in a pattern similar to that seen with coeliac disease patient sera (**IV**; Table 2). Cultured fibroblasts exhibited positive staining both intra- and extracellularly with mAb-tTGs (**IV**; Figure 1E).

tTG and tTG+gliadin immunised mouse sera were positive for IgG-class antibodies, producing the same coeliac-type tissue staining pattern as detected with untreated coeliac disease patient sera in all tissues studied. By comparison, gliadin-immunised mouse sera were negative for EmA and ARA but contained antibodies reacting with the smooth muscle cell cytoplasm. Fibronectin was observed to be co-distributed with tTG (**IV**; Table 2).

In a double immunofluorescent test the mAb-tTG CUB 7402 showed positivity identical to the staining pattern of untreated coeliac disease patient sera in all tissues studied (**IV**; Figures 1A, 1B, 1G, 1H). When these two staining images were merged, complete overlapping was demonstrated (**IV**; Figures 1C, 1I). Overlapping was also observed with other tTG antibodies. In cultured Wharton's jelly-derived fibroblasts overlapping was demonstrated both intra- and extracellularly (**IV**; Figure F). By comparison, no overlap was observed with non-coeliac sera and any of the tTG antisera used (**IV**; Figure 1J) and with coeliac disease patient sera and gliadin-immunised mouse sera (**IV**; Figure 1K). The staining images of coeliac disease patient sera and fibronectin antibodies overlapped only partially around jejunal capillaries (**IV**; Figure 1L).

Antibody competition studies

An excess amount of IgA-class EmA positive coeliac disease patient sera (undiluted or diluted 1:2) completely blocked the positive tissue staining of mAb-tTG CUB 7402 (dilution 1:1000, or more) on human umbilical cord. IgA-deficient coeliac disease patient serum positive for IgG-class EmA had the same effect. Excess amount of mAb-tTG CUB 7402 resulted in only partial blocking of the IgA-class EmA of coeliac disease patient sera. The effect was seen only in the intensity of fluorescence but not in the titre.

Effect of potassium thiocyanate treatment on antigen and endomysial staining

KSCN treatment completely abolished both IgA-and IgG-class endomysial stainings (**IV**: Figure 4A). It also reduced or completely abolished the fluorescence of all tTG antibodies used (**IV**: Table 4). In contrast, the fluorescence of non-coeliac sera and fibronectin antibodies remained essentially unchanged.

Absorption studies

The results here showed that fibroblast-derived antigen proteins bound most of the IgA, inducing the expression of EmA and R_1-type ARA (**I**: Table 1), while tTG bound most of the IgA responsible for tTG antibodies (**IV**: Table 3). Furthermore, tTG clearly reduced the positivity for ARA and to a lesser extent for EmA. In contrast, crude gliadin almost completely bound the IgA responsible for AGA, but had only little or no effect on EmA, ARA and tTG antibodies.

Discussion

Human umbilical cord-based antibody tests

It was demonstrated here that human umbilical cord tissue is a proper substitute for rat tissues and monkey oesophagus in indirect immunofluorescence tests when screening for coeliac disease. It would appear that the use of human umbilical cord as substrate enables large-scale detection of coeliac disease; it is inexpensive and readily available tissue, and does not involve ethical issues which might limit its long-term use.

Consistent with published results (Volta et al. 1995, Sacchetti et al. 1996, Bottaro et al. 1997, Kolho and Savilahti 1997, Sategna-Guidetti et al. 1997) our findings show that IgA-class EmA on human umbilical cord is particularly sensitive (93%) for the diagnosis of coeliac disease, with the reservation, however, that while the test appeared to be extremely effective in detecting 98% of children with untreated coeliac disease; among adults with untreated coeliac disease only 85% sensitivity was achieved. Although IgA-class EmA have proved to be highly efficient in detecting untreated coeliac disease, there are also contradictory reports. According to Rostami et al. (1999) EmA testing has only limited value in screening programs for coeliac disease as many coeliacs were initially negative for the antibodies.

Recent demonstrations of the high specificity of the IgA-class EmA test have raised the issue of whether small-bowel biopsies are needed in IgA-class EmA-positive patients with a clinical suspicion of coeliac disease (Valdimarsson et al. 1996b, Del Rosario et al. 1998). This high specificity of the test (99%) was indeed confirmed by the present results. However, the case may be different when screening applies to unselected populations with a low disease prevalence or individuals with vague or no symptoms.

The results of Feighery et al. (1998a) emphasise that a combination of clinical, histological and serological criteria are required for the effective diagnosis of coeliac disease in asymptomatic patients. Exclusive reliance on histology or serology will thus, result in failure to make a diagnosis in a significant number of patients.

IgA-class EmA titres decline on a gluten-free diet. This would imply that IgA-class EmA determination is a reliable serological test for monitoring initial response to a gluten-free diet. The reappearance of IgA-class EmA during gluten challenge is consequently a good marker of the reactivation of the disease.

In the present study it was empirically established that in the measurement of IgG-class EmA the umbilical cord of preterm infants is preferable to that of full-term, markedly reducing the intense background staining. The sensitivity of the IgG-class EmA test was significantly lower (12%) than that of the IgA-class EmA test. Its specificity, however, was 100%. Moreover, the IgG-class EmA test identified one coeliac patient negative for other IgA- and IgG-class antibodies. IgG-class EmA would also seem to be reliable in IgA-deficient subjects, detecting 100% of patients with coeliac disease and this deficiency. The frequency of IgA deficiency in coeliac patients has been reported to represent a 10-16 increase over that in the population at large (Cataldo et al. 1998). Only the determination of IgG-class antibodies can identify these coeliac patients with concomitant IgA deficiency.

Our results show that apart from reticulin fibrils in the connective tissue surrounding muscle cells in the cord blood vessels, coeliac patient serum IgA also binds to cytoplasmic components of cells in Wharton’s jelly. Likewise, Feighery et al. (Feighery et al. 1998b) demonstrated EmA reactivity with cells in Wharton’s jelly. Fibroblasts are the major cell type in this gelatinous, mucopolysaccharide-rich material surrounding the cord blood vessels. In the present study cultured Wharton’s jelly-derived fibroblasts

exhibited cytoplasmic staining with coeliac patient sera similar to the staining pattern of human foetal lung fibroblasts (Marttinen and Mäki 1993). Moreover, our previously identified human foetal lung fibroblast-derived autoantigen proteins (Marttinen and Mäki 1993) bound to IgA-class EmA as well as to ARA and monkey oesophagus EmA in absorption studies. This would indicate that the mesenchymal-derived Wharton's jelly fibroblasts possess molecules recognised by coeliac disease patient serum IgA. This insight further prompted us to develop a whole-cell ELISA method using Wharton's jelly-derived fibroblasts as antigen for the diagnosis of coeliac disease. As the current results show, all untreated coeliac disease children tested were positive for fibroblast autoantibodies in the novel whole-cell ELISA. The test is, however, somewhat too laborious and time-consuming for use in clinical practice.

Tissue transglutaminase autoantibodies

The identification of tTG as the endomysial autoantigen (Dieterich et al. 1997) has provided one more tool for coeliac disease screening. The present findings bear out the conception that tTG can be used as antigen in an ELISA test (Dieterich et al. 1997). From a technical point of view, it emerged that a better coating was achieved when calcium was present in the coating buffer. It has been shown that in the presence of calcium, tTG tends to form homoaggregates (Wihelm et al. 1996). More importantly, however, calcium probably induces a conformational change in tTG, making it possible for tTG antibodies to recognise and bind it (Fésus and Laki 1977). As both coeliac patients and controls have increased antibody titres to food proteins (Scott et al. 1990), we decided here to avoid the use of bovine serum albumin as a blocking agent.

Our data showed that the measurement of IgA-class tTG antibodies is highly sensitive (95%) and specific (94%) for coeliac disease, indicating that an ELISA designed to

detect tTG antibodies has considerable potential in the diagnosis of coeliac disease. In agreement with the present results, Dieterich et al. (1998) reported IgA-class tTG antibodies in 104 out of 106 (98%) and Troncone et al. (1999) in 44 out of 48 (92%) patients with untreated coeliac disease. However, in recent studies by Bazzigaluppi et al. (1999) and Lock et al. (1999) only 85% sensitivity was achieved. Further, the former group reported that a radiobinding assay using *in vitro* transcribed and translated transglutaminase as antigen showed higher sensitivity and specificity than ELISA using guinea pig transglutaminase as antigen.

IgA-class tTG antibodies were negative or lower in patients on a gluten-free diet than in untreated coeliacs. This finding is in line with the observation that EmA and ARA titres tend to disappear after gluten withdrawal, and that after the reintroduction of gluten-containing food tTG antibodies are again produced, indicating the reactivation of the disease.

While our results demonstrate that an IgG response to tTG is found in almost all patients with coeliac disease, this reaction was also detected in control subjects. In contrast to our findings, Troncone et al. (1999) reported that the sensitivity of the IgG-class tTG antibody ELISA test was low (21%), but that the specificity was as high as 98%. In the present study the diagnostic value of the IgG-class tTG antibody test was nevertheless assessed, since it found all IgA-deficient coeliac patients who had no detectable IgA anti-tTG.

Comparison of antibody tests

Our data show that in clinical practice, human umbilical cord, rat tissues and monkey oesophagus can be used alternatively as substrates, since the titres of the tests correlate well. The classical IgA-class ARA proved a reliable marker of coeliac disease. The IgA-

class EmA test, however, detected more adults with untreated coeliac disease than did the IgA-class ARA test. IgA-class EmA also had a lower false-positive rate than IgA-class ARA: three out of 346 control patients (specificity 99%) were positive for IgA-class EmA, and 20 of them (specificity 94%) had positive IgA-class ARA titres. Thus, human tissue as substrate would seem to be more sensitive and specific for coeliac disease than rat tissue. However, it is worth noting that combination of IgA-class EmA with IgA-class ARA offers additional diagnostic advantages detecting more patients with untreated coeliac disease than either test alone. It has been shown, moreover, that the positive ARA in patients with normal mucosal villous architecture indicate later mucosal deterioration and gluten sensitivity (Collin et al. 1993, Kaukinen et al. 1998). It can thus, be hypothesised that overt villous atrophy had not yet developed in all 20 IgA-class ARA-positive patients, and that they may be gluten-sensitive.

The concordance rate between the IgA-class tTG antibody and the IgA-class EmA tests was very high (93%). Similar high frequencies of IgA-class tTG antibodies and IgA-class EmA were found in patients with untreated coeliac disease both here and in other recent studies (Dieterich et al. 1998, Troncone et al. 1999). IgA-class tTG antibodies also appear to be as sensitive as R_1-type ARA. Such results suggest that tTG antibody ELISA could replace the semiquantitative, somewhat cumbersome and subjective indirect immunofluorescent tests on sections of human umbilical cord or rat tissues and might allow the simple, economical and rapid screening of large populations for silent coeliac disease. However, the ELISA test based on commercial guinea pig tTG antigen was less specific than the EmA test using human umbilical cord as antigen, especially in adults. It is possible that the ELISA test using guinea pig antigen may measure antibodies which are not specific for coeliac disease, and thus, the use of human tTG could provide increased specificity. The recent cloning of human tTG might represent a

further advance towards reliable diagnosis of coeliac disease (Lampasona et al. 1998, Fasano 1999).

The results of the present study confirm that neither IgA- nor IgG-class AGA are specific for coeliac disease. Moreover, the predictive ability of the IgA-class AGA test was clearly lower than that of the IgA-class EmA, ARA and tTG antibody tests. On the other hand, in study **II** high IgA-class AGA titres were found in untreated coeliacs negative for IgA-class EmA and ARA, which would suggest that the combined determination of high titre IgA-class AGA and IgA-class EmA and ARA obviously gives an excellent prediction of mucosal damage.

It should be recognised that a subgroup of coeliac patients do not show any serological abnormalities and will require small-bowel biopsy. In study **II** 4% of untreated adult coeliacs remained negative in all three serological screening tests (EmA, ARA and AGA) used. It has indeed been suggested that the antibodies in question co-exist with the appearence of severe villous atrophy, and that coeliac disease patients with milder mucosal changes evince lower sensitivity in serology (Rostami et al. 1999).

Relationships between endomysial, reticulin and tissue transglutaminase autoantibodies

In the present study the staining patterns of the mAb-tTG on both rodent and primate tissues resembled those obtained with coeliac patient sera. Further, in the double immunostaining studies mAb-tTG showed complete overlapping reactivity with coeliac patient sera. These results strongly support the findings of Dieterich et al. (1997) that tTG is the predominant autoantigen in coeliac disease. The current observations are also in accord with recent results obtained by Lock et al. (1999), who demonstrated that IgA-class tTG antibodies purified from coeliac patient sera showed a clear and convincing

endomysial and reticulin staining pattern. The results here suggest that tTG autoantibody reactivity accounts for EMA, ARA and jejunal antibody appearances.

Antigen removal and reconstitution experiments showed that both mAb-tTG and coeliac patient sera were similarly dependent on the presence of tTG. Antibody competition and absorption studies further indicated that tTG can be the target for the autoantibodies detected in coeliac disease. However, after the affinity chromatography experiments the residual EmA/ARA staining activity was seen to persist in the effluent from the tTG column. Thus, the possibility remains that further autoantigenic epitopes exist which are not related to tTG (Uhlig et al. 1998, Lock et al. 1999).

Although tTG is primarily an intracellular enzyme, highly expressed in the enterocytes of the villi (Thomazy and Fésus 1989), fibroblasts (Sharp et al. 1988) and endothelial cells (Greenberg et al. 1987), a significant amount of it can be detected associated with the extracellular matrix after wounding (Upchurch et al. 1991). The possibility of secretion of tTG by an alternative pathway cannot, however, be excluded (Aeschlimann and Paulsson 1991). The present study demonstrated that tTG is present intracellulary and also in the extracellular matrix of cultured human umbilical cord Wharton's jelly-derived fibroblasts. Interestingly, these fibroblasts were shown to secrete a coeliac IgA- and tTG-positive extracellular reticular network, a finding in agreement with the observation of Berman et al. (Berman et al. 1978) that the fibroblast-produced extracellular reticular network is silver-staining-positive. Hence, the results of the present study indicate that tTG is also extracellulary located in normal human tissues.

Fibronectin, a protein associated with the extracellular matrix, is one of the specific substrates for tTG activity (Fésus and Arato 1986). It has been described as a component of reticulin, and the distribution of fibronectin in the reticular network is very similar to that of bound antibodies (Unsworth et al. 1982). The present results show that

fibronectin is codistributed with tTG in both rodent and primate tissues. Nonetheless, fibronectin is not the antigen recognised by EmA/ARA (Unsworth et al. 1984). The present results confirm this.

Summary and conclusions

In the present study human umbilical cord was used as antigen in immunofluorescence assay, and the viability of the test to detect coeliac disease was evaluated. Novel ELISA methods were established to measure coeliac disease-specific antibodies to human umbilical cord-derived fibroblasts, and to tTG. The study population included 276 patients with untreated coeliac disease, 346 controls and 14 coeliac patients with IgA-deficiency. For comparative purposes ARA and monkey oesophagus EmA were examined by immunofluorescence techniques and AGA with ELISA. To study the tissue distribution of tTG coeliac and non-coeliac patient sera, mAb-tTGs and sera from mice parenterally immunised against tTG, tTG complexed with gliadin, or gliadin were used in indirect immunofluorescence and double-staining studies. In addition, KSCN-extraction, antibody competition and affinity chromatography studies were performed to study further the relationship between tTG and tissue autoantibodies.

IgA-class EmA proved highly sensitive (258 of 276) and specific (343 of 346) in detecting coeliac disease. Comparison of IgA-class EmA test to the ARA test (sensitivity 92%, specificity 94%) and to the monkey oesophagus EmA test (sensitivity 100%, specificity 83%) showed good correlation. Although the IgA-class AGA test yielded a lower sensitivity (83%) and specificity (77%) than the IgA-class EmA and ARA tests, high AGA titres were found in eight out of 12 patients negative for EmA and ARA.

The novel whole-cell fibroblast autoantibody ELISA detected all 27 coeliacs tested, giving a sensitivity of 100%; with a specificity of 82% (54 of 66). The test correlated better with the EmA than with the AGA test. The calcium-treated tTG antibody ELISA proved to be highly sensitive, 129 of 136 (95%) tested untreated coeliac disease patients

were positive. Thirteen out of 207 disease control patients had elevated titres (specificity 94%). The concordance rate between the IgA-class tTG antibody ELISA and IgA-class EmA tests was high (93%). The predictive ability of the new tTG antibody ELISA test was superior to that of the AGA test.

In selective IgA deficiency the IgG-class EmA, ARA and tTG autoantibody tests seem to work well. All 14 untreated coeliac patients with selective IgA deficiency were positive for these autoantibodies.

The tTG antibody staining patterns here were seen to be identical to those with coeliac patient sera in all tissues studied. Cultured human umbilical cord-derived fibroblasts exhibited both cytoplasmic and extracellular matrix staining. Double staining with coeliac patient serum IgA and mAb-tTG showed complete overlapping. KSCN-extraction of tTG from the tissue sections abolished the staining patterns, and they were restored after re-addition of tTG. Patient sera blocked the staining of mAb-tTG, and tTG effectively absorbed EmA/ARA from coeliac patient sera.

I conclude that human umbilical cord replaces perfectly rat and monkey tissues when using the indirect immunofluorescent test for detecting coeliac disease-specific autoantibodies. The present study shows that coeliac disease self-epitopes are present also in human umbilical cord and that the Wharton's jelly-derived fibroblasts indeed express molecules detectable by coeliac disease patient sera IgA. These fibroblasts could be used as antigen in the novel whole-cell ELISA test. Further, the findings here constitute evidence of the value of a combined search for IgA-class EmA, ARA and high-titre AGA.

The present data further confirm that tTG is a major autoantigen in coeliac disease. In clinical practice the ELISA designed for the detection of tTG antibodies provides an alternative possibility for a sensitive, specific and objective serological screening for the

diagnosis and follow-up of coeliac disease. It was also established that tTG is distributed in both rodent and primate tissues identically to the extracellular endomysial/reticulin/jejunal staining pattern of coeliac patient sera. The current results indicate that EmA, ARA and jejunal autoantibodies detect tTG in different tissues.

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References

Acerini, C.L., Ahmed, M.L., Ross, K.M., Sullivan, P.B., Bird, G., and Dunger, D.B. (1998). Coeliac disease in children and adolescents with IDDM: clinical charasteristics and response to gluten-free diet. *Diabet. Med.* 15, 38-44.

Aeschlimann, D., and Paulsson, M. (1991). Cross-linking of laminin-nidogen complexes by tissue transglutaminase. *J Biol Chem* 266, 15308-17.

Aine, L., Mäki, M., Collin, P., and Keyriläinen, O. (1990). Dental enamel defects in celiac disease. *J Oral Pathol Med* 19, 241-5.

Arranz, E., and Ferguson, A. (1993). Intestinal antibody pattern of celiac disease: occurrence in patients with normal jejunal biopsy histology. *Gastroenterology* 104, 1263-72.

Ascher, H., Lanner, A., and Kristiansson, B. (1990). A new laboratory kit for anti-gliadin IgA at diagnosis and follow-up of childhood celiac disease. *J Pediatr Gastroenterol Nutr* 10, 443-50.

Ascher, H., Krantz, I., and Kristiansson, B. (1991). Increasing incidence of coeliac disease in Sweden. *Arch Dis Child* 66, 608-11.

Auricchio, S., Mazzacca, G., Tosi, R., Visakorpi, J.K., Mäki, M., and Polanco, I. (1988). Coeliac disease as a familial condition: identification of asymptomatic coeliac patients within family groups. *Gastroenterol Int* 1, 25-31.

Bardella, M.T., Molteni, N., Prampolini, L., Giunta, A.M., Baldassarri, A.R., Morganti, D., and Bianchi, P.A. (1994). Need for follow up in coeliac disease. *Arch Dis Child* 70, 211-3.

Bardella, M.T., Fraquelli, M., Quatrini, M., Molteni, N., Bianchi, P., and Conte, D. (1995). Prevalence of hypertransaminasemia in adult celiac patients and effect of gluten-free diet. *Hepatology* 22, 833-6.

Bazzigaluppi, E., Lampasona, V., Barera, G., Venerando, A., Bianchi, C., Chiumello, G., Bonifacio, E., and Bosi, E. (1999). Comparison of tissue transglutaminase-specific antibody assays with established antibody measurements for coeliac disease. *J Autoimmunity* 12, 51-6.

Berman, J., Stoner, G., Dawe, C., Rice, J., and Kingsbury, E. (1978). Histochemical demonstration of collagen fibers in ascorbic-acid-fed cell cultures. *In Vitro* 14, 675-85.

Bjarnason, I., Peters, T.J., and Veall, N. (1983). A persistent defect in intestinal permeability in coeliac disease demonstrated by a 51Cr-labelled EDTA absorption test. *Lancet* 1, 323-5.

Bode, S., and Gudmand-Hoyer, E. (1994). Evaluation of the gliadin antibody test for diagnosing coeliac disease. *Scand J Gastroenterol* 29, 148-52.

Boismenu, R., and Havran, W.L. (1994). Modulation of epithelial growth by intraepithelial gamma/delta T cells. *Science* 266,1253-5.

Bottaro, G., Volta, U., Spina, M., Rotolo, N., Sciacca, A., and Musumeci, S. (1997). Antibody pattern in childhood celiac disease. *J Pediatr Gastroenterol Nutr* 24, 559-62.

Brandtzaeg, P., Bosnes, V., Halstensen T.S., Scott, H., Sollid, L.M., Valnes, K.N. (1989). T lymphocytes in human gut epithelium express preferentially the α/β antigen receptor and are often CD45/UCHL1 positive. *Scand J Immunol* 30, 123-8.

Bruce, S.E., Bjarnason, I., and Peters, T.J. (1985). Human jejunal transglutaminase: demonstration of activity, enzyme kinetics and substrate specificity with special relation to gliadin and coeliac disease. *Clin Sci* 68, 573-9.

Bürgin-Wolff, A., Bertele, R.M., Berger, R., Gaze, H., Harms, H.K., Just, M., Khanna, S., Schurmann, K., Signer, E., and Tomovic, D. (1983). A reliable screening test for childhood celiac disease: fluorescent immunosorbent test for gliadin antibodies. A prospective multicenter study. *J Pediatr* 102, 655-60.

Bürgin-Wolff, A., Gaze, H., Hadziselimovic, F., Huber, H., Lentze, M J., Nussle, D., and Reymond, B.-C. (1991). Antigliadin and antiendomysium antibody determination for coeliac disease. *Arch Dis Child* 66, 941-7.

Cataldo, F., Marino, V., Ventura, A., Bottaro, G., and Corazza, G.R. (1998). Prevalence and clinical features of selective immunoglobulin A deficiency in coeliac disease: an Italian multicentre study. Italian Society of Paediatric Gastroenterology and Hepatology (SIGEP) and "Club del Tenue" Working Groups on Coeliac Disease. *Gut* 42, 362-5.

Catassi, C., Rätsch, I. M., Fabiani, E., Rossini, M., Bordicchia, F., Candela, F., Coppa, G.V., and Giorgi, P.L. (1994). Coeliac disease in the year 2000: exploring the iceberg. *Lancet* 343, 200-3.

Cavell, B., Stenhammar, L., Ascher, H., Danielsson, L., Dannaeus, A., Lindberg, T., and Lindquist, B. (1992). Increasing incidence of childhood coeliac disease in Sweden. Results of a national study. *Acta Paediatr* 81, 589-92.

Challacombe, D.N., and Bayliss, J.M. (1980). Childhood coeliac disease is disappearing. *Lancet* 2, 1360.

Chorzelski, T.P., Sulej, J., Tchorzewska, H., Jablonska, S., Beutner, E.H., and Kumar, V. (1983). IgA class endomysium antibodies in dermatitis herpetiformis and coeliac disease. *Ann N Y Acad Sci* 420, 325-34.

Collin, P., Salmi, J., Hällström, O., Oksa, H., Oksala, H., Mäki, M., and Reunala, T. (1989). High frequency of coeliac disease in adult patients with type-I diabetes. *Scand J Gastroenterol* 24, 81-4.

Collin, P., Hällström, O., Mäki, M., Viander, M., and Keyriläinen, O. (1990). Atypical coeliac disease found with serologic screening. *Scand J Gastroenterol* 25, 245-50.

Collin, P., Pirttilä, T., Nurmikko, T., Somer, H., Erilä, T., and Keyriläinen, O. (1991). Celiac disease, brain atrophy and dementia. *Neurology* 41, 372-5.

Collin, P., Mäki, M., Keyriläinen, O., Hällström, O., Reunala, T., and Pasternack, A. (1992a). Selective IgA deficiency and coeliac disease. *Scand J Gastroenterol* 27, 367-71.

Collin, P., Korpela, M., Hällström, O., Viander, M., Keyriläinen, O., and Mäki, M. (1992b). Rheumatic complaints as a presenting symptom in patients with coeliac disease. *Scand J Rheumatol* 21, 20-3.

Collin, P., Helin, H., Mäki, M., Hällström, O., and Karvonen, A.-L. (1993). Follow-up of patients positive in reticulin and gliadin antibody tests with normal small bowel biopsy findings. *Scand J Gastroenterol* 28, 595-8.

Collin, P., and Mäki, M. (1994). Associated disorders in coeliac disease: clinical aspects. *Scand J Gastroenterol* 29, 769-75.

Collin, P., Reunala, T., Pukkala, E., Laippala, P., Keyriläinen, O., and Pasternack, A. (1994a). Coeliac disease - associated disorders and survival. *Gut* 35, 1215-8.

Collin, P., Salmi, J., Hällström, O., Reunala, T., and Pasternack, A. (1994b). Autoimmune thyroid disorders and coeliac disease. *Eur J Endocrinol* 130, 137-40.

Collin, P., Vilska, S., Heinonen, P.K., Hällström, O., and Pikkarainen, P. (1996). Infertility and coeliac disease. *Gut* 39, 382-4.

Collin, P., Reunala, T., Rasmussen, M., Kyrönpalo, S., Pehkonen, E., Laippala, P., and Mäki, M. (1997). High incidence and prevalence of adult coeliac disease. Augmented diagnostic approach. *Scand J Gastroenterol* 32, 1129-33.

Cooke, W.T., and Holmes, G.K.T. (1984). *Coeliac disease.* Churchill Livingstone, Edinburgh.

Cooper, B.T., Holmes, G.K.T., and Cooke, W.T. (1978). Coeliac disease and immunological disorders. *BMJ* 1, 537-9.

Corazza, G.R., Frisoni, M., Treggiari, E.A., Valentini, R.A., Filipponi, C., Volta, U., and Gasbarrini, G. (1993). Subclinical celiac sprue. Increasing occurrence and clues to its diagnosis. *J Clin Gastroenterol* 16, 16-21.

Corazza, G.R., Andreani, M.L., Venturo, N., Bernardi, M., Tosti, A., and Gasbarrini, G. (1995). Celiac disease and alopecia areata: report of a new association. *Gastroenterology* 109, 1333-7.

Corazza, G.R., Andreani, M.L., Biagi, F., Bonvicini, F., Bernardi, M., and Gasbarrini, G. (1996). Clinical, pathological, and antibody pattern of latent celiac disease: report of three adult cases. *Am J Gastroenterol* 91, 2203-7.

Cronin, C.C., and Shanahan, F. (1997). Insulin-dependent diabetes mellitus and coeliac disease. *Lancet* 349, 1096-7.

Csizmadia, C.G.D.S., Mearin, M.L., von Blomberg, B.M.E., Brand, R., and Verloove-Vanhorick, S.P. (1999). An iceberg of childhood coeliac disease in the Netherlands. *Lancet* 353, 813-4.

Del Rosario, M., Fitzgerald, J.F., Chong, S.K., Croffie, J.M., and Gupta, S.K. (1998). Further studies of anti-endomysium and anti-gliadin antibodies in patients with suspected celiac disease. *J Pediatr Gastroenterol Nutr* 27, 191-5.

DeMarchi, M., Carbonara, A., Ansaldi, N., Santini, B., Barbera, C., Borelli, I., Rossini, P., and Rendine, S. (1983). HLA-DR3 and DR7 in coeliac disease: immunogenetic and clinical aspects. *Gut* 24, 706-12.

Dieterich, W., Ehnis, T., Bauer, M., Donner, P., Volta, U., Riecken, E.O., and Schuppan, D. (1997). Identification of tissue transglutaminase as the autoantigen of celiac disease. *Nature Med* 3, 797-801.

Dieterich, W., Laag, E., Schopper, H., Volta, U., Ferguson, A., Gillett, H., Riecken, E.O., and Schuppan, D. (1998). Autoantibodies to tissue transglutaminase as predictors of celiac disease. *Gastroenterology* 115, 1317-21.

Dossetor, J.F., Gibson, A.A., and McNeish, A.S. (1981). Childhood coeliac disease is disappearing. *Lancet* 1, 322-3.

Douglas, J.G., Gillon, J., Logan, R.F., Grant, I.W., and Crompton, G.K. (1984). Sarcoidosis and coeliac disease: an association? *Lancet* 2, 13-5.

Eade, O.E., Lloyd, R.S., Lang, C., and Wright, R. (1977). IgA and IgG reticulin antibodies in coeliac disease and non-coeliac patients. *Gut* 18, 991-3.

Ek, J., Albrechtsen, D., Solheim, B.G., and Thorsby, E. (1978). Strong association between the HLA-Dw3-related B cell alloantigen-DRw3 and coeliac disease. *Scan J Gastroenterol* 13, 229-33.

Falchuk, Z.M., Rogentine, F.N., and Strober, W. (1972). Predominance of histocompatibility antigen HLA-A8 in patients with gluten-sensitive enteropathy. *J Clin Invest* 51, 1602-6.

Falchuk, Z.M., and Strober, W. (1974). Gluten-sensitive enteropathy. Synthesis of antigliadin antibody in vitro. *Gut* 15, 947-52.

Fasano, A. (1996). Where have all the American celiacs gone? *Acta Paediatr* 85(Suppl 412), 20-4.

Fasano, A. (1999). Tissue transglutaminase: the holy grail for the diagnosis of celiac disease, at least? *J Pediatr* 134, 134-5.

Feighery, C., Weir, D.G., Whelan, A., Willoughby, R., Youngprapakorn, S., Lynch, S., O'Morain, C., McEany, P., and O'Farrelly, C. (1998a). Diagnosis of gluten-sensitive enteropathy: is exclusive reliance on histology appropriate? *Eur J Gastroenterol Hepatol* 10, 919-25.

Feighery, C., Abuzakouk, M., Liddy, C., Jackson, J., Whelan, A., Willoughby, R., Cronin, C., and Kumararatne, D.S. (1998b). Endomysial antibody detection using human umbilical cord tissue as substrate: reactivity of cells in Wharton's jelly. *Br J Biomed Sci* 55, 107-10.

Ferguson, A., and Murray, D. (1971). Quantitation of intraepithelial lymphocytes in human jejunum. *Gut* 12, 988-94.

Ferguson, M.M., Wray, D., Carmichael, H.A., Russell, R.I., and Lee, F.D. (1980). Coeliac disease associated with recurrent aphthae. *Gut* 21, 223-6.

Ferguson, A., Blackwell, J.N., and Barnetson, R.S. (1987). Effects of additional dietary gluten on the small-intestinal mucosa of volunteers and of patients with dermatitis herpetiformis. *Scand J Gastroenterol* 22, 543-9.

Ferguson, A., Arranz, E., and O'Mahony, S. (1993). Clinical and pathological spectrum of coeliac disease - active, silent, latent, potential. *Gut* 34, 150-1.

Fernandez-Arguero, M., Clerici, N., Polanco, I., Escobar, H., Figueredo, M.A., and de la Concha, E. G. (1995). HLA-DQ alleles and susceptibility to celiac disease in Spanish children. *Tissue Antigens* 45, 145-7.

Ferreira, M., Davies, S. L., Butler, M., Scott, D., Clark, M., and Kumar, P. (1992). Endomysial antibody: is it the best screening test for coeliac disease? *Gut* 33, 1633-7.

Fésus, L., and Laki, K. (1977). Two antigenic sites of tissue transglutaminase. *Biochemistry* 16, 4061-6.

Fésus, L., and Arato, G. (1986). Quantitation of tissue tranglutaminase by a sandwich ELISA system. *J Immunol Methods* 94, 131-6.

Fraser-Reynolds, K.A., Butzner, J.D., Stephure, D.K., Trussell, R.A., and Scott, R.B. (1998). Use of immunoglobulin A-antiendomysial antibody to screen for celiac disease in North American children with type 1 diabetes. *Diabetes Care* 21, 1985-9.

Gale, L., Wimalaratna, H., Brotodiharjo, A., and Duggan, J.M. (1997). Down's syndrome is strongly associated with coeliac disease. *Gut* 40, 492-6.

Gardner, M.J., and Altman, D.G. (1989). *Statistics with confidence*. BMJ, London.

Gee, S. (1888). On the coeliac disease. *St Bart Hosp Rep* 24, 17-20.

George, E. K., Mearin, M.L., Bouquet, J., von Blomberg B. M., Stapel, S.O., van Elburg R.M., and de Graaf E. A. (1996). High frequency of celiac disease in Down syndrome. *J Pediatr* 128, 555-7.

Gjertsen, H.A., Sollid, L.M., Ek, J., Thorsby, E., and Lundin, K.E.A. (1994). T cells from the peripheral blood of coeliac disease patients recognize gluten antigens when presented by HLA-DR, -DQ, or –DP molecules. *Scand J Immunol* 39, 567-74.

Gobbi, G., Bouquet, F., Greco, L., Lambertini, A., Tassari, A., Ventura, A., and Zaniboni, M. (1990). Coeliac disease, epilepsy and cerebral calcifications. *Lancet* 340, 439-443.

Greco, L., Corazza, G., Babron, M.-C., Clot, F., Fulchignoni-Lataud, M.-C., Percopo, S., Zavattari, P., Bouguerra, F., Dib, C., Tosi, R., Troncone, R., Ventura, A., Mantavoni, W., Magazzu, G., Gatti, R.,

Lazzari, R., Giunta, A., Perri, F., Iacono, G., Cardi, E., de Virgiliis, S., Cataldo, F., De Angelis, G., Musumeci, S., Ferrari, R., Balli, F., Bardella, M.-T., Volta, U., Catassi, C., Torre, G., Eliaou, J.-F., Serre, J.-L., and Clerget-Darpoux, F. (1998). Genome search in celiac disease. *Am J Hum Genet* 62, 669-75.

Greco, L., Mäki, M., Di Donato, F., and Visakorpi, J.K. (1992). Epidemiology of coeliac disease in Europe and the Mediterranean area. A summary report on the multicentric study by the European Society for Paediatric Gastroenterology and Nutrition. In: *Common food intolerances 1: Epidemiology of coeliac disease*. Auricchio, S. and Visakorpi, J. K. eds. pp. 25-44. Karger, Basel.

Greenberg, C.S., Achyuthan, K.E., Borowitz, M.J., and Shuman, M.A. (1987). The transglutaminase in vascular cells and tissues could provide an alternate bathway for fibrin stabilization. *Blood* 70, 702-9.

Grodzinsky, E., Hed, J., Lieden, G., Sjogren, F., and Strom, M. (1990). Presence of IgA and IgG antigliadin antibodies in healthy adults as measured by micro-ELISA. Effect of various cutoff levels on specificity and sensitivity when diagnosing coeliac disease. *Int Arch Allergy Appl Immunol* 92, 119-23.

Grodzinsky, E., Franzen, L., Hed, J., and Ström, M. (1992). High prevalence of celiac disease in healthy adults revealed by antigliadin antibodies. *Ann Allergy* 69, 66-70.

Hadjivassiliou, M., Grunewald, R.A., Chattopadhyay, A.K., Davies-Jones, G.A.B., Gibson, A., Jarrat, J.A., Kandler, R.H., Lobo, A., Powell, T., and Smith, C.M.L. (1998). Clinical, radiological, neuropscyhological, and neuropathological characteristics of gluten ataxia. *Lancet* 352, 1582-5.

Hällström, O. (1989). Comparison of IgA-class reticulin and endomysium antibodies in coeliac disease and dermatitis herpetiformis. *Gut* 30, 1225-32.

Halstensen, T.S., Scott, H.,Brandtzaeg, P. (1989). Intraepithelial T cells of the TcRγ/δ+CD8- and Vδ1/Jδ1+ phenotypes are increased in celiac disease. Scand J Immunol 30, 665-72.

Halstensen, T.S., Hvatum, M., Scott, H., Fausa, O., and Brandtzaeg, P. (1992). Association of subepithelial deposition of activated complement and immunoglobulin G and M response to gluten in celiac disease. *Gastroenterology* 102, 751-9.

Halttunen, T., Marttinen, A., Rantala, I., Kainulainen, H., and Mäki, M. (1996). Fibroblasts and transforming growth factor beta induce organization and differentation of T84 human epithelial cells. *Gastroenterology* 111, 1252-62.

Halttunen, T., and Mäki, M. (1999). Serum immunoglobulin A from patients with celiac disease inhibits human T84 intestinal crypt epithelial cell differentation. *Gastroenterology* 116, 566-72.

Hardman, C.M., Garioch, J.J., Leonard, J.N., Thomas, H.J.W., Walker, M.M., Lortan, J.E., Lister, A., and Fry, L. (1997). Absence of toxicity of oats in patients with dermatitis herpetiformis. *New England J Med* 337, 1884-7.

Hodgson, H.J., Davies, R.J., and Gent, A.E. (1976). Atopic disorders and adult coeliac disease.*Lancet* 1, 115-7.

Holm, K.H. (1993). Correlation of HLA-DR alleles to jejunal mucosal morphology in healthy first-degree relatives of coeliac disease patients. *Eur J Gastroenterol Hepatol* 5, 35-9.

Holmes, G.K.T., Prior, P., Lane, M.R., Pope, D., and Allan, R.N. (1989). Malignancy in coeliac disease - effect of a gluten free diet. *Gut* 30, 333-8.

Houlston, R.S., Tomlinson, I.P., Ford, D., Seal, S., Marossy, A.M., Ferguson, A., Holmes, G.K., Hosie, K.B., Howdle, P.D., Jewell, D.P., Godkin, A., Kerr, G.D., Kumar, P., Logan, R.F., Love, A.H., Johnston, S., Marsh, M.N., Mitton, S., O'Donoghue, D., Roberts, A., Walker-Smith, J.A., and Stratton, M.F. (1997). Linkage analysis of candidate regions for coeliac disease genes. *Hum Mol Genet* 6, 1335-9.

Howdle, P.D., Ciclitira, P.J., Simpson, F.G., and Losowsky, M.S. (1984). Are all gliadins toxic in coeliac disease? An in vitro study of alpha, beta, gamma, and w gliadins. *Scand J Gastroenterol* 19, 41-7.

Iltanen, S., Collin, P., Korpela, M., Holm, K., Partanen, J., Polvi, A., and Mäki, M. (1999a). Celiac disease and markes of celiac disease latency in patients with primary Sjögren's syndrome. *Am J Gastroenterol* 94, 1042-6.

Iltanen, S., Holm, K., Partanen, J., Laippala, P., and Mäki, M. (1999b). Increased density of jejunal γδ+ T cells in patients having normal mucosa - marker of operative autoimmune mechanisms? *Autoimmunity* 29, 179-87.

Jacobsen, M.B., Fausa, O., Elgjo, K., and Schrumpf, E. (1990). Hepatic lesions in adult coeliac disease. *Scand J Gastroenterol* 25, 656-62.

Janatuinen, E.K., Pikkarainen, P.H., Kemppainen, T.A., Kosma, V.-M., Järvinen, R.M.K., Uusitupa, M.I.J., and Julkunen, R.J.K. (1995). A comparison of diets with and without oats in adults with celiac disease. *N Engl J Med* 333, 1033-7.

Johnston, S.D., Watson, R.G.P., McMillan, S.A., Sloan, J., and Love, A.H.G. (1997). Prevalence of coeliac disease in Northern Ireland. *Lancet* 350, 1370.

Kagnoff, M.F., Austin, R.K., Hubert, J.J., Bernardin, J.E., and Kasarda, D.D. (1984). Possible role for a human adenovirus in the pathogenesis of celiac disease. *J Exp Med* 160, 1544-57.

Kárpáti, S., Török, E., and Kosnai, I. (1986). IgA class antibody against human jejunum in sera of children with dermatitis herpetiformis. *J Invest Dermatol* 87, 703-6

Kárpáti, S., Bürgin-Wolff, A., Krieg, T., Meurer, M., Stolz, W., and Braun-Falco, O. (1990). Binding to human jejunum of serum IgA antibody from children with coeliac disease. *Lancet* 336, 1335-8.

Kárpáti, S., Stolz, W., Meurer, M., Krieg, T., and Braun-Falco, O. (1991). Extracellular binding sites of IgA anti-jejunal antibodies on normal small bowel detected by indirect immunoelectronmicroscopy. *J Invest Dermatol* 96, 228-33.

Kárpáti, S., Meurer, M., Stolz, W., Bürgin-Wolff, A., Braun-Falco, O., and Krieg, T. (1992). Ultrastructural binding sites of endomysium antibodies from sera of patients with dermatitis herpetiformis and coeliac disease. *Gut* 33, 191-3.

Kaukinen, K., Collin, P., Holm, K., Karvonen, A.-L., Pikkarainen, P., and Mäki, M. (1998). Small bowel mucosal inflammation in reticulin or gliadin antibody-positive patients without villous atrophy. *Scand J Gastroenterol* 33, 944-9.

Kaukinen, K., Collin, P., Holm, K., Rantala, I., Vuolteenaho, N., Reunala, T., and Mäki, M. (1999). Wheat starch-containing gluten-free flour products in the treatment of coeliac disease and dermatitis herpetiformis. A long-term follow-up study. *Scand J Gastroenterol* 34, 164-9.

Kelly, J., O'Farrelly, C., Rees, J.P., Feighery, C., and Weir, D.G. (1987). Humoral response to alpha gliadin as serological screening test for coeliac disease. *Arch Dis Child* 62, 469-73.

Kelly, C.P., Feighery, C.F., Gallagher, R.B., and Weir, D.G. (1990). Diagnosis and treatment of gluten-sensitive enteropathy. *Adv Intern Med* 35, 341-63.

Kerttula, T.O., Collin, P., Polvi, A., Korpela, M., Partanen, J., and Mäki, M. (1996). Distinct immunological features of Finnish Sjögren's syndrome patients with the alleles HLA DRB1*0301, and DQA1*0501 DQB1*0201. Alterations in circulating T cell receptor gd subsets. *Arthritis Rheum* 39, 1733-9.

Keuning, J.J., Pena, A.S., van Leeuwen, A., van Hooff, J.P., and van Rood, J.J. (1976). HLA-DW3 associated with coeliac disease. *Lancet* 1, 506-8.

Kilander, A.F., Dotevall, G., Fallstrom, S.P., Gillberg, R.E., Nilsson, L.A., and Tarkowski, A. (1983). Evaluation of gliadin antibodies for detection of coeliac disease. *Scand J Gastroenterol* 18, 377-83.

Kokkonen, J., Similä, S., and Vuolukka, P. (1982). The incidence of coeliac disease and pyloric stenosis in Northern Finland. *Ann Clin Res* 14, 123-8.

Kolho, K.-L., and Savilahti, E. (1997). IgA endomysium antibodies on human umbilical cord: an excellent diagnostic tool for celiac disease in childhood. *J Pediatr Gastroenterol Nutr* 24, 563-7.

Kolho, K.-L., Färkkilä, M.A., and Savilahti, E. (1998). Undiagnosed coeliac disease is common in Finnish adults. *Scand J Gastroenterol* 33, 1280-3.

Korponay-Szabo, I.R., Kovacs, J.B., Czinner, A., Goracz, G., Vamos, A., and Szabo, T. (1999). High prevalence of silent celiac disease in preschool children screened with IgA/IgG antiendomysium antibodies. *J Pediatr Gastroenterol Nutr* 28, 26-30.

Kumar, P. J., Walker-Smith, J., Milla, P., Harris, G., Colyer, J., and Halliday, R. (1988). The teenage coeliac: follow up study of 102 patients. *Arch Dis Child* 63, 916-20.

Kumar, V., Lerner, A., Valeski, J. E., Beutner, E.H., Chorzelski, T.P., and Rossi, T. (1989). Endomysial antibodies in the diagnosis of celiac disease and the effect of gluten on antibody titers. *Immunol Invest* 18, 533-44.

Kurokawa, M., Lynch, K., and Podolsky, D.K. (1987). Effects of growth factors on an intestinal epithelial cell line: transforming growth factor beta inhibits proliferation and stimulates differentation. *Biochem Biophys Res Commun* 142, 775-82.

Ladinser, B., Rossipal, E., and Pittschieler, K. (1994). Endomysium antibodies in coeliac disease: an improved method. *Gut* 35, 776-8.

Lähdeaho, M.-L., Lehtinen, M., Rissa, H.R., Hyöty, H., Reunala, T., and Mäki, M. (1993). Antipeptide antibodies to adenovirus E1b protein indicate enhanced risk of celiac disease and dermatitis herpetiformis. *Int Arch Allergy Immunol* 101, 272-6.

Lampasona, V., Bazzigaluppi, E., Barera, G., and Bonifacio, E. (1998). tissue transglutaminase and combined screening for coeliac disease and type 1 diabetes-associated autoantibodies. *Lancet* 352, 1192-3.

Lancaster-Smith, M.J., Perrin, J., Swarbrick, E.T., and Wright, J.T. (1974). Coeliac disease and autoimmunity. *Postgrad Med J* 50, 45-8.

Lerner, A., Kumar, V., and Iancu, T.C. (1994). Immunological diagnosis of childhood coeliac disease: comparison between antigliadin, antireticulin and antiendomysial antibodies. *Clin Exp Immunol* 95, 78-82.

Levenson, S.D., Austin, R.K., Dietler, M.D., Kasarda, D.D., and Kagnoff, M.F. (1985). Specificity of antigliadin antibody in celiac disease. *Gastroenterology* 89, 1-5.

Littlewood, J.M., Crollick, A.J., and Richards, I.D.G. (1980). Childhood coeliac disease is disappearing. *Lancet* 2, 1359-60.

Lock, R..J., Pitcher, M.C., Unsworth, D.J. (1999a). IgA anti-tissue transglutaminase as a diagnostic marker of gluten sensitive enetropathy. *J Clin Pathol* 52, 274-7.

Lock, R.J., Gilmour, J.E.M., and Unsworth, D.J. (1999b). Anti-transglutaminase, anti-endomysium and anti-R1-reticulin autoantibodies - the antibody trinity of coeliac disease. *Clin Exp Immunol* 116, 258-62.

Logan, R.F.A., Tucker, G., Rifkind, E.A., Heading, R.C., and Ferguson, A. (1983). Changes in clinical features of coeliac disease in adults in Edinburgh and the Lothians 1960-79. *BMJ* 286, 95-7.

Lundin, K.E.A., Scott, H., Hansen, T., Paulsen, G., Halstensen, T. S., Fausa, O., Thorsby, E., and Sollid, L.M. (1993). Gliadin-specific, HLA-DQ(A1*0501, B1*0201) restricted T cells isolated from the small intestinal mucosa of celiac disease patients. *J Exp Med* 178, 87-96.

MacDonald, T.T., and Spencer, J. (1988). Evidence that activated mucosal T cells play a role in the pathogenesis of enteropathy in human small intestine. *J Exp Med* 167, 1341-9.

Magalhaes, A.F., Peters, T.J., and Doe, W.F. (1974). Studies on the nature and significance of connective tissue antibodies in adult coeliac disease and Chron's disease. *Gut* 15, 284-8.

Mäki, M., Hällström, O., Vesikari, T., and Visakorpi, J.K. (1984a). Evaluation of a serum IgA-class reticulin antibody test for the detection of childhood celiac disease. *J Pediatr* 105, 901-5.

Mäki, M., Hällström, O., Huupponen, T., Vesikari, T., and Visakorpi, J.K. (1984b). Increased prevalence of coeliac disease in diabetes. *Arch Dis Child* 59, 739-42.

Mäki, M., Kallonen, K., Lähdeaho, M.-L., and Visakorpi, J.K. (1988a). Changing pattern of childhood coeliac disease in Finland. *Acta Paediatr Scand* 77, 408-12.

Mäki, M., Hällström, O., Verronen, P., Reunala, T., Lahdeaho, M.-L., Holm, K., and Visakorpi, J.K. (1988b). Reticulin antibody, arthritis, and coeliac disease in children. *Lancet* 1, 479-80.

Mäki, M., Lahdeaho, M.-L., Hällström, O., Viander, M., and Visakorpi, J.K. (1989). Postpubertal gluten challenge in coeliac disease. *Arch Dis Child* 64, 1604-7.

Mäki, M., Holm, K., Koskimies, S., Hällström, O., and Visakorpi, J.K. (1990). Normal small bowel biopsy followed by coeliac disease. *Arch Dis Child* 65, 1137-41.

Mäki, M., Hällström, O., and Marttinen, A. (1991a). Reaction of human non-collagenous polypeptides with coeliac disease autoantibodies. *Lancet* 338, 724-5.

Mäki, M., Holm, K., Collin, P., and Savilahti, E. (1991b). Increase in γ/δ T cell receptor bearing lymphocytes in normal small bowel mucosa in latent coeliac disease. *Gut* 32, 1412-4.

Mäki, M., Holm, K., Lipsanen, V., Hällström, O., Viander, M., Collin, P., Savilahti, E., and Koskimies, S. (1991c). Serological markers and HLA genes among healthy first-degree relatives of patients with coeliac disease. *Lancet* 338, 1350-3.

Mäki, M., Holm, K., Ascher, H., and Greco, L. (1992). Factors affecting clinical presentation of coeliac disease: Role of type and amount of gluten-containing cereals in the diet. In: *Common food intolerances 1: Epidemiology of coeliac disease.* Auricchio, S. and Visakorpi, J. K., eds. pp. 76-82. Karger, Basel.

Mäki, M. (1995). The humoral immune system in coeliac disease. *Baillieres Clin Gastroenterol* 9, 231-49.

Mäki, M., and Collin, P. (1997). Coeliac disease. *Lancet* 349, 1755-9.

Marsh, M.N. (1992). Gluten, major histocompatibility complex, and the small intestine. A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). *Gastroenterology* 102, 330-54.

Marttinen, A., and Mäki, M. (1993). Purification of fibroblast-derived celiac disease autoantigen molecules. *Ped Res* 34, 420-3.

Mawhinney, H., and Lowe, A.H.G. (1975). Anti-reticulin antibody in jejunal juice in coeliac disease. *Clin Exp Immunol* 21, 394-8.

Mayer, M., Greco, L., Troncone, R., Grimaldi, M., and Pansa, G. (1989). Early prediction of relapse during gluten challenge in childhood celiac disease. *J Pediatr Gastroenterol Nutr* 8, 474-9.

Mayer, M., Greco, L., Troncone, R., Auricchio, S., and Marsh, M.N. (1991). Compliance of adolescents with coeliac disease with a gluten free diet. *Gut* 32, 881-5.

Mazure, R., Vazquez, H., Gonzalez, D., Mautalen, C., Pedreira, S., Boerr, L., and Bai, J.C. (1994). Bone mineral affection in asymptomatic adult patients with celiac disease. *Am J Gastroenterol* 89, 2130-4.

McMillan, S.A., Haughton, D.J., Biggart, J.D., Edgar, J.D., Porter, K.G., and McNeill, T.A. (1991). Predictive value for coeliac disease of antibodies to gliadin, endomysium, and jejunum in patients attending for jejunal biopsy. *BMJ* 303, 1163-5.

Mearin, M.L., Biemond, I., Pena, A.S., Polanco, I., Vazquez, C., Schreuder, G.T., de Vries, R.R., and van Rood, J.J. (1983). HLA-DR phenotypes in Spanish coeliac children: their contribution to the understanding of the genetics of the disease. *Gut* 24, 532-7.

Meloni, G., Dore, A., Fanciulli, G., Tanda, F., and Bottazzo, G.F. (1999). Subclinical coeliac disease in schoolchildren from northern Sardinia. *Lancet* 353, 37.

Michalski, J.P., McCombs, C.C., Arai, T., Elston, R.C., Cao, T., McCarthy, C.F., and Stevens, F.M. (1996). HLA-DR, DQ genotypes of celiac disease patients and healthy subjects from the West of Ireland.*Tissue Antigens* 47, 127-33.

Molberg, Ø., Kett, K., Scott, H., Thorsby, E., Sollid, L.M., and Lundin, K.E.A. (1997). Gliadin specific, HLA DQ2-restricted T cells are commonly found in small intestinal biopsies from coeliac disease patients, but not from controls. *Scand J Immunol* 46, 103-8.

Molberg, , Ø., Mcadam, S.N., Korner, R., Quarsten, H., Kristiansen, C., Madsen, L., Fugger, L., Scott, H., Noren, O., Roepstorff, P., Lundin, K.E.A., Sjöström, H., and Sollid, L.M. (1998). Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. *Nature Med* 4, 713-7.

Molteni, N., Bardella, M.T., and Bianchi, P.A. (1990). Obstetric and gynecological problems in women with untreated celiac sprue. *J Clin Gastroenterol* 12, 37-9.

Mustalahti, K., Collin, P., Sievänen, H., Salmi, J., and Mäki, M. (1999). Osteopenia in patients with clinically silent coeliac disease warrants screening. *Lancet* 354, 744-5.

Mylotte, M., Egan-Mitchell, B., Fottrell, P.F., McNicholl, B., and McCarthy, C.F. (1974). Family studies in coeliac disease. *Q J Med* 43, 359-369.

Not, T., Horvath, K., Hill, I.D., Partanen, J., Hammed, A., Magazzu, G., and Fasano, A. (1998). Celiac disease risk in the USA, high prevalence of antiendomysium antibodies in healthy blood donors. *Scand J Gastroenterol* 33, 494-8.

Nunes, I., Gleizes, P.E., Metz, C.N., and Rifkin, D.B. (1997). Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol* 136, 1151-63.

Oberhuber, G., Vogelsang, H., Stolte, M., Muthenthaler, S. Kummer, A.J., and Radaszkiewicz, T. (1996). Evidence that intestinel intraepithelial lymphocytes are activated cytotoxic T cells in celiac disease but not in giardiasis. *Am J Pathol* 148, 1351-7.

O'Farrelly, C., Kelly, J., Hekkens, W., Bradley, B., Thompson, A., Feighery, C., and Weir, D.G. (1983). Alpha gliadin antibody levels: a serological test for coeliac disease. *BMJ* 286, 2007-10.

O'Mahony, S., Arranz, E., Barton, J.R., and Ferguson, A. (1991). Dissociation between systemic and mucosal humoral immune responses in coeliac disease. *Gut* 32, 29-35.

Page, S.R., Lloyd, C.A., Hill, P.G., Peacock, I., and Holmes, G.K.T. (1994). The prevalence of coeliac disease in adult diabetes mellitus. *Q J Med* 87, 631-7.

Pena, A.S., Mann, D.L., Hague, N.E., Heck, J.A., van Leeuwen, H.A., van Rood, J.J., and Strober, W. (1978). Genetic basis of gluten-sensitive enteropathy. *Gastroenterology* 75, 230-5.

Pender, S.L.F., Tickle, S.P., Docherty, A.J.P., Howie, D., Wathen, N.C., and MacDonald, T. (1997). A major role for matrix metalloproteinases in T cell injury in the gut. *J Immunol* 158, 1582-90.

Petronzelli, F., Bonamico, M., Ferrante, P., Grillo, R., Mora, B., Mariani, P., Apollonio, I., Gemme, G., and Mazzilli, M C. (1997). Genetic contribution of the HLA region to the familial clustering of coeliac disease. *Ann Hum Genet* 1997, 307-17.

Pettersson, A., Sjöberg, K., Lernmark, Å., and Eriksson, S. (1993). HLA genotypes in coeliac disease and healthy individuals carrying gliadin antibodies. *Eur J Gastroenterol Hepatol* 5, 445-50.

Picarelli, A., Maiuri, L., Frate, A., Greco, M., Auricchio, S., and Londei, M. (1996). Production of antiendomysial antibodies after in-vitro glaidin challenge af small intestine biopsy samples from patients with coeliac disease. *Lancet* 348, 1065-7.

Ploski, R., Ek, J., Thorsby, E., and Sollid, L.M. (1993). On the HLA-DQ(alpha 1*0501, beta 1*0201)-associated susceptibility in celiac disease: a possible gene dosage effect of DQB1*0201. *Tissue Antigens* 41, 173-7.

Polanco, I., Biemond, I., van Leeuwen, A., Schreuder, I., Meera Khan, P., Guerrero, J., D'Amaro, J., Vazques, C., van Rood, J.J., and Pena, A.S. (1981). Gluten sensitive enteropathy in Spain: genetic and environmental factors. In: *The genetics of coeliac disease*. McConell, R. B. ed. pp. 211-34. MTP Press, Lancaster.

Polvi, A., Arranz, E., Fernandez-Arquero, M., Collin, P., Mäki, M., Sanz, A., Calvo, C., Maluenda, C., Westman, P., de la Concha, E.G., and Partanen, J. (1998). HLA-DQ2-negative celiac disease in Finland and Spain. *Hum Immunol* 59, 169-75.

Rensch, M.J., Merenich, J.A., Lieberman, M., Long, B.D., Davis, D.R., and McNally, P.R. (1996). Gluten-sensitive enteropathy in patients with insulin-dependent diabetes mellitus. *Ann Intern Med* 124, 564-7.

Reunala, T., Kosnai, I., Karpati, S., Kuitunen, P., Török, E., and Savilahti, E. (1984). Dermatitis herpetiformis: jejunal findings and skin response to gluten-free diet. *Arch Dis Child* 59, 517-522.

Reunala, T., Salmi, J., and Karvonen, J. (1987). Dermatitis herpetiformis and celiac disease associated with Addison's disease. *Arch Dermatol* 123, 930-2.

Reunala, T., Collin, P., Holm, K., Pikkarainen, P., Miettinen, A., Vuolteenaho, N., and Mäki, M. (1998). Tolerance to oats in dermatitis herpetiformis. *Gut* 43, 490-3.

Risch, N. (1987). Assessing the role of HLA-linked and unlinked determinants of disease. *Am J Hum Genet* 40, 1-14.

Rizzetto, M., and Doniah, D. (1973). Types of 'reticulin' antibodies detected in human sera by immunofluorescence. *J Clin Pathol* 26, 841-51.

Roberts, S.J., Smith, A.L., West, A.B., Wen, L., Findly, R.C., Owen, M.J., and Hayday, A.C. (1996). T-cell alpha beta + and gamma delta + deficient mice display abnormal but distinct phenotypes toward a natural, widespread infection of the intestinal epithelium. *Proc Natl Acad Sci USA* 93, 11774-9.

Rossi, T.M., Albini, C.H., and Kumar, V. (1993). Incidence of celiac disease identified by the presence of serum endomysial antibodies in children with chronic diarrhea, short stature, or insulin-dependent diabetes mellitus. *J Pediatr* 123, 262-4.

Rostami, K., Kerckhaert, J., Tiemessen, R., Meijer, J.W.R., and Mulder, C.J.J. (1999). The relationship between anti-andomysium antibodies and villous atrophy in coeliac disease using both monkey and human substrate.*Eur J Gastroenterol Hepatol* 11, 439-42.

Sacchetti, L., Ferrajolo, A., Salerno, G., Esposito, P., Lofrano, M. M., Oriani, G., Micillo, M., Paparo, F., Troncone, R., Auricchio, S., and Salvatore, F. (1996). Diagnostic value of various serum antibodies detected by diverse methods in childhood celiac disease. *Clin Chem* 42, 1838-42.

Sategna-Guidetti, C., Grosso, S., Pulitano, R., Benaduce, E., Dani, F., and Carta, Q. (1994). Celiac disease and insulin-dependent diabetes mellitus. Screening in an adult population. *Dig Dis Sci* 39, 1633-7.

Sategna-Guidetti, C., Grosso, S., Bruno, M., and Grosso, S.B. (1995). Comparison of serum anti-gliadin, anti-endomysium, and anti-jejunum antibodies in adult celiac sprue. *J Clin Gastroenterol* 20, 17-21.

Sategna-Guidetti, C., Grosso, S.B., Bruno, M., and Grosso, S. (1997). Is human umbilical cord the most suitable substrate for the detection of endomysium antibodies in the screening and follow-up of coeliac disease? *Eur J Gastroenterol Hepatol* 9, 657-60.

Savilahti, E., Pelkonen, P., and Visakorpi, J.K. (1971). IgA deficiency in children. A clinical study with special reference to intestinal findings. *Arch Dis Child* 46, 665-70.

Savilahti, E. (1972). Intestinal immunoglobulins in children with coeliac disease. *Gut* 13, 958-64.

Savilahti, E., Viander, M., Perkkio, M., Vainio, E., Kalimo, K., and Reunala, T. (1983). IgA antigliadin antibodies: a marker of mucosal damage in childhood coeliac disease. *Lancet* 1, 320-2.

Savilahti, E., Simell, O., Koskimies, S., Rilva, A., and Åkerblom, H.K. (1986). Celiac disease in insulin-dependent diabetes mellitus. *J Pediatr* 108, 690-3.

Savilahti, E., Arato, A., and Verkasalo, M. (1990). Intestinal γ/δ receptor-bearing T lyphocytes in celiac disease and inflammatory bowel diseases in children. Constant increase in celiac disease. *Pediatr Res* 28, 579-81.

Scott, H., and Brandtzaeg, P. (1989). Gluten IgA antibodies and coeliac disease. *Lancet* 1, 382-3.

Scott, H., Fausa, O., Ek, J., Valnes, K., Blystad, L., and Brandtzaeg, P. (1990). Measurements of serum IgA and IgG activities to dietary antigens. A prospective study of the diagnostic usefulness in adult coeliac disease. *Scand J Gastroenterol* 25, 287-92.

Seah, P.P., Fry, L.L., Rossiter, M.A., Hoffbrand, A.V., and Holborow, E. (1971). Anti-reticulin antibodies in childhood coeliac disease. *Lancet* I, 834-6.

Sharp, G., Grindley, H., Lorand, L., and Peters, T.J. (1988). Immunofluorescent localization of transglutaminase in rat small intestine. *Cell Biochem Funct* 6, 137-41.

Sher, K.S., Jayanthi, V., Probert, C.S., Stewart, C.R., and Mayberry, J.F. (1994). Infertility, obstetric and gynaecological problems in coeliac sprue. *Dig Dis* 12, 186-90.

Similä, S., and Kokkonen, J. (1990). Coexistence of celiac disease and Down syndrome. *Am J Ment Retard* 95, 120-2.

Snook, J.A., de Silva, H.J., and Jewell, D.P. (1989). The association of autoimmune disorders with inflammatory bowel disease. *Q J Med* 72, 835-40.

Sollid, L.M., Markussen, G., Ek, J., Gjerde, H., Vartdal, F., and Thorsby, E. (1989). Evidence for a primary association of celiac disease to a particular HLA-DQ α/β heterodimer. *J Exp Med* 169, 345-50.

Sollid, L.M., and Thorsby, E. (1993). HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* 105, 910-22.

Sollid, L.M., Molberg, Ø., McAdam, S., and Lundin, K.E.A. (1997). Autoantibodies in coeliac disease: tissue transglutaminase - guilt by association. *Gut* 41, 851-2.

Spencer, J., Isaacson, P.G., Diss, T.C., MacDonald, T.T. (1989). Expression of disulfide-linked and non-disulfide-linked forms of the T cell receptor γ/δ heterodimerin human intraepithelial lymphocytes. *Eur J Immunol* 19;1335-8.

Spurkland, A., Sollid, L. M., Polanco, I., Vartdal, F., and Thorsby, E. (1992). HLA-DR and DQ genotypes of celiac disease patients serologically typed to be non-DR3 or non-DR5/7. *Hum Immunol* 35, 188-92.

Srinivasan, U., Leonard, N., Jones, E., Kasarda, D.D., Weir, D.G., O'Farrelly, C., and Feighery, C. (1996). Absence of oats toxicity in adult coeliac disease. *BMJ* 313, 1300-1.

Ståhlberg, M.-R., Savilahti, E., and Viander, M. (1986). Antibodies to gliadin by ELISA as a screening test for childhood celiac disease. *J Pediatr Gastroenterol Nutr* 5, 726-9.

Stenhammar, L., Fallstrom, S.P., Jansson, G., Jansson, U., and Lindberg, T. (1986). Coeliac disease in children of short stature without gastrointestinal symptoms. *Eur J Pediatr* 145, 185-6.

Stern, M., Adenäuer, M., Keilmann, C., and Wäscher, I. (1997). Standardization of screening tests. In: *Coeliac disease. Proceedings of the seventh international symposium on coeliac disease. September 5-7, 1996, Tampere, Finland.* Mäki, M., Collin, P., and Visakorpi, J. K. eds. pp. 35-46, Tampere.

Stevens, F. M., Egan-Mitchell, B., Cryan, E., McCarthy, C.F., and McNicholl, B. (1987). Decreasing incidence of coeliac disease. *Arch Dis Child* 62, 465-8.

Stokes, P.L., Asquith, P., Holmes, G.K., MacIntosh, P., and Cooke, W.T. (1972). Histocompatibility antigens associated with adult coeliac disease. *Lancet* 2, 162-4.

Sturgess, R.P., Ellis, H.J., and Ciclitira, P.J. (1991). Cereal chemistry, molecular biology, and toxicity in coeliac disease. *Gut* 32, 1055-60.

Swinson, C.M., and Levi, A.J. (1980). Is coeliac disease underdiagnosed? *BMJ* 281, 1258-60.

Talal, A.H., Murray, J.A., Goeken, J.A., and Sivitz, W.I. (1997). Celiac disease in an adult population with insulin-dependent diabetes mellitus: use of endomysial antibody testing. *Am J Gastroenterol* 92, 1280-4.

Thomazy, V., and Fésus, L. (1989). Differential expression of tissue tranglutaminase in human cell: an immunohistochemical study. *Cell Tissue Res* 2, 215-24.

Tosi, R., Vismara, D., Tanigaki, N., Ferrara, G.B., Cicimarra, F., Buffolano, W., Follo, D., and Auricchio, S. (1983). Evidence that celiac disease is primarily associated with a DC locus allelic specificity. *Clin Immunol Immunopathol* 28, 395-404.

Trejdosiewics, L.K., and Howdle, P.D. (1995). T-cell responses and cellular immunity in coeliac disease. *Baillieres Clin Gastroenterol* 9, 251-72.

Troncone, R., and Ferguson, A. (1991). Anti-gliadin antibodies. *J Pediatr Gastroenterol Nutr* 12, 150-8.

Troncone, R. (1995). Latent coeliac disease in Italy. *Acta Paediatr* 84, 1252-7.

Troncone, R., Maurano, F., Rossi, M., Micillo, M., Greco, L., Auricchio, R., Salerno, G., Salvatore, F., and Sacchetti, L. (1999). IgA antibodies to tissue transglutaminase: an effective diagnostic test for celiac disease. *J Pediatr* 134, 166-71.

Tuckova, L., Tlaskalova, H.-H., Farre, M.A., Karska, K., Rossmann, P., Kolinska, J., and Kocna, P. (1995). Molecular mimicry as a possible cause of autoimmune reactions in celiac disease? Antibodies to gliadin cross-react with epitopes on enterocytes. *Clin Immunol Immunopathol* 74, 170-6.

Uhlig, H., Osman, A.A., Tanev, I.D., Viehweg, J., and Mothes, T. (1998). Role of tissue transglutaminase in gliadin binding to reticular extracellular matrix in relation to coeliac disease autoantibodies. *Autoimmunity* 28, 185-95.

Uibo, O., Uibo, R., Kleimola, V., Jögi, T., and Mäki, M. (1993). Serum IgA anti-gliadin antibodies in an adult population sample. High prevalence without celiac disease. *Dig Dis Sci* 38, 2034-7.

Unsworth, D.J., Scott, D.L., Almond, T.J., Beard, H.K., Holborow, E.J., and Walton, K.W. (1982). Studies on reticulin I: Serological and immunohistological investigation of the occurrence of collagen type III, fibronectin and the non-collagenous glycoprotein of Pras and Glynn in reticulin. *Br J Pathol* 63, 154-66.

Unsworth, D.J., Walker, S.-J.A., and Holborow, E.J. (1983). Gliadin and reticulin antibodies in childhood coeliac disease. *Lancet* 1, 874-5.

Unsworth, D.J., Scott, D.L., Walton, K.W., Walker, S.-J.A., and Holborow, E.J. (1984). Failure of R1 type anti-reticulin antibody to react with fibronectin, collagen type III or the non-collagenous reticulin component (NCRC). *Clin Exp Immunol* 57, 609-13.

Unsworth, D.J., Leonard, J.N., Hobday, C.M., Griffiths, C.E., Powles, A.V., Haffenden, G.P., and Fry, L. (1987). Gliadins bind to reticulin in a lectin-like manner. *Arch Dermatol Res* 279, 232-5.

Unsworth, D.J., and Brown, D.L. (1994). Serological screening suggests that adult coeliac disease is underdiagnosed in the UK and increases the incidence by up to 12%. *Gut* 35, 61-4.

Upchurch, H.F., Conway, E., Patterson, M.K., Jr, and Maxwell, MD. (1991). Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme. *J Cell Physiol* 149, 375-82.

Valdimarsson, T., Löfman, O., Toss, G., and Ström, M. (1996a). Reversal of osteopenia with diet in adult coeliac disease. *Gut* 38, 322-7.

Valdimarsson, T., Franzen, L., Grodzinsky, E., Skogh, T., and Ström, M. (1996b). Is small bowel biopsy necessary in adults with suspected celiac disease and IgA anti-endomysium antibodies? 100% positive predictive value in adults. *Dig Dis Sci* 41, 83-7.

Valeski, J.E., Kumar, V., Beutner, E.H., Lerner, A., and Chorzelski, T.P. (1990). Immunology of celiac disease: tissue and species specificity of endomysial and reticulin antibodies. *Int Arch Allergy Appl Immunol* 93, 1-7.

Ventura, A., Magazzu, G., and Greco, L. (1999). Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. *Gastroenterology* 117, 297-303.

Verkasalo, M., Kuitunen, P., Leisti, S., and Perheentupa, J. (1978). Growth failure from symptomless celiac disease. A study of 14 patients. *Helv Pediatr Acta* 33, 489-95.

Visakorpi, J.K., Immonen, P., and Kuitunen, P. (1967). Malabsorption syndrome in childhood. *Acta Paediatr Scand* 56, 1-9.

Visakorpi, J.K., Kuitunen, P., and Pelkonen, P. (1970). Intestinal malabsorption: a clinical study of 22 children over 2 years of age. *Acta Paediatr Scand* 59, 273-80.

Visakorpi, J.K., and Mäki, M. (1994). Changing clinical features of coeliac disease. *Acta Paediatr Suppl* 83, 10-3.

Vogelsang, H., Genser, D., Wyatt, J., Lochs, H., Ferenci, P., Granditsch, G., and Penner, E. (1995). Screening for celiac disease: a prospective study on the value of noninvasive tests. *Am J Gastroenterol* 90, 394-8.

Volta, U., Molinaro, N., Fratangelo, D., and Bianchi, F. B. (1990). IgA subclass antibodies to gliadin in serum and intestinal juice of patients with coeliac disease. *Clin Exp Immunol* 80, 192-5.

Volta, U., Molinaro, N., Fusconi, F., Cassani, F., and Bianchi, F. B. (1991). IgA antiendomysial antibody test. A step forward in celiac disease screening. *Dig Dis Sci* 36, 752-6

Volta, U., Molinaro, N., De Franceschi, L., Fratangelo, D., and Bianchi, F. B. (1995). IgA anti-endomysial antibodies on human umbilical cord tissue for celiac disease screening. Save both money and monkeys. *Dig Dis Sci* 40, 1902-5.

Volta, U., De Franceschi, L., Lari, F., Molinaro, N., Zoli, M., and Bianchi, F.B. (1998). Coeliac disease hidden by cryptogenic hypertransaminasemia. *Lancet* 352, 26-9.

van de Wal, Y., Kooy, Y., van Veelen, P., Pena, S., Mearin, L., Papadopoulos, G., and Koning, F. (1998). Cutting edge: Selective deamination by tissue transglutaminase strongly enhances gliadin-specific T cell reactivity. *J Immunol* 161, 1585-8.

Walker-Smith, J.A., Guandalini, S., Schmitz, J., Shmerling, D.H., and Visakorpi, J.K. (1990). Revised criteria for diagnosis of coeliac disease. *Arch Dis Child* 65, 909-11.

Weijers, H.A., Lindquist, B., Anderson, C.M., Rey, J., Shmerling, D.H., Visakorpi, J.K., Hadorn, B., and Gruttner, R. (1970). Diagnostic cirteria in coeliac disease. *Acta Paediatr Scand* 59, 461-3.

Weinstein, W.M. (1974). Latent celiac sprue. *Gastroenterology* 66, 489-93.

Whelan, A., Willoughby, R., and Weir, D. (1996). Human umbilical vein endothelial cells: a new easily available source of endomysial antigens. *Eur J Gastroenterol and Hepatol* 8, 961-6.

Wihelm, B., Meihardt, A., and Seitz, J. (1996). Transglutaminases: purification and activity assays. *J Chromatogr B Biomed Appl* 684, 163-77.

Yiannakou, J.Y., Dell'Olio, D., Saaka, M., Ellis, H.J., Rosen-Bronson, S., Dumonde, D.C., and Ciclitira, P.J. (1997). Detection and characterisation of anti-endomysial antibody in coeliac disease using human umbilical cord. *Int Arch Allergy Immunol* 112, 140-4.

Zhong, F., McCombs, C.C., Olson, J.M., Elston, R.C., Stevens, F.O., McCarthy, C.F., and Michalski, J.P. (1996). An autosomal screen for genes that predispose to celiac disease in the western counties of Ireland. *Nature Genet* 14, 329-33.

Original publications