SEMMELWEIS UNIVERSITY, PH.D. DOCTORAL SCHOOL

MOLECULAR MEDICINE

(MULTIDISCIPLINARY MEDICINE)

Leader: Prof. Dr. József Mandl

Ph.D. thesis

written by

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Programme title:	Basics of Human Molecular Genetics and Genetic Diagnostics
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Role of tissue (type 2) transglutaminase in the pathogenesis and diagnosis of coeliac disease and dermatitis herpetiformis

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Budapest, 2002.

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ABBREVIATIONS

aa ..... amino acid
Ab, Abs ..... antibody, antibodies
AGA ..... anti-gliadin antibody
ARA ..... anti-reticulin antibody
AI ..... autoimmune
AU ..... arbitrary unit(s)
AUC ..... area under the ROC curves
BC_a ..... bias-corrected and accelerated
BSA ..... bovine serum albumin
CD ..... coeliac disease
CMV ..... cytomegalovirus
cDNA ..... complementary desoxyribonucleic acid
CI ..... confidence interval
cM ..... centimorgan (1 cM=1% recombination frequency)
DH ..... dermatitis herpetiformis
EATL ..... enteropathy-associated T-cell lymphoma
EB4.2 ..... erythrocyte protein band 4.2
EMA ..... endomysium antibody
ESPGAN ..... European Society for Paediatric Gastroenterology and Nutrition
FXIIIa ..... factor XIII a subunit
GCH ..... gluten challenge
GFD ..... gluten-free diet
GI ..... gastrointestinal
gpTGc ..... guinea pig TGc
GSD ..... gluten-sensitive disease (see page 9 for definition)
GSE ..... gluten-sensitive enteropathy (see page 9 for definition)
h ..... hour(s)
HLA ..... human leukocyte group A
hTGc ..... human TGc
kb ..... kilobase(s)
kD ..... kilodalton(s)
MAb ..... monoclonal antibody
min ..... minute(s)
MHC ..... major histocompatibility complex
OD ..... optical density
PBS ..... phosphate-buffered saline
PMSF ..... phenylmethanesulfonyl fluoride
RBC ..... red blood cell
ROC ..... receiver operating characteristic
r_S ..... Spearman's correlation coefficient
SDS-PAGE ..... sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS ..... Tris-buffered saline
TET ..... 50 mM Tris/HCl containing 10 mM EDTA and 0.1% Tween 20

TG ..... transglutaminase
TGc ..... tissue (cellular, type 2) transglutaminase
TGc-/- ..... TGc knockout (homozygote for a disrupted TGc gene)
TGe ..... epidermal (type 3) transglutaminase
TGe-/- ..... TGe knockout (homozygote for a disrupted TGe gene)
TGF-â ..... transforming growth factor â
TGk ..... keratinocyte (type 1) transglutaminase
TGp ..... prostate (type 4) transglutaminase
TGx ..... transglutaminase X (type 5)
TGy ..... transglutaminase Y (type 6)
TGz ..... transglutaminase Z (type 7)

1. ÖSSZEFOGLALÁS

A szöveti (2-es típusú) transzglutamináz szerepe a coeliakia és a dermatitis herpetiformis pathogenesisében és diagnosztikájában

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A humán molekuláris genetika és géndiagnosztika alapjai
Budapest, 2002.

Bevezetés. Néhány évvel ezelõtt a szöveti (2-es típusú) transzglutaminázt (TGc) coeliakiában (CD) és dermatitis herpetiformisban (DH) szenvedõ betegek keringõ IgA ellenanyagainak, az ún. endomysium ellenanyagok fõ autoantigénjeként azonosították. Ezen ellenanyagok kimutatására egy tengerimalac TGc-n alapuló ELISA tesztet is kifejlesztettek. Célunk az volt, hogy igazoljuk e vizsgálati módszer jelentõségét a CD és a DH diagnosztikájában, a szûrésben és a betegek követésében. Az eredeti ELISA-t továbbfejlesztettük humán rekombináns antigén használatával, és összehasonlítottuk már létezõ szerológiai tesztekkel (tengerimalac TGc ELISA és endomysium antitest teszt). Vizsgáltuk ezen kívül a módszer specificitását valamint az ellenanyagok esetleges elõfordulását más betegségekben is.

Módszerek. Az egészséges kontrolloktól valamint coeliakiában vagy dermatitis herpetiformisban szenvedõ betegektõl származó savók mellett olyan betegek szérumát is teszteltük, akik más (elsõsorban autoimmun vagy egyéb gastrointestinalis) rendellenességekben szenvedtek. A keringõ IgA autoantitestek kimutatására olyan ELISA-t készítettünk, melyhez májból kivont tengerimalac- és rekombinánsan elõállított humán TGc-t használtunk kálcium-aktivált formában. Az endomysium-ellenes IgA antitesteket majom nyelõcsövön végzett indirekt immunfluoreszcenciával detektáltuk.

Eredmények. A humán TGc-t tiszta és aktív enzimként állítottuk elõ. Egészséges személyekkel összehasonlítva mind a tengerimalac, mind a humán TGc alapú ELISA specificitása és szenzitivitása magas volt kezeletlen CD-s és DH-s betegekben. Jelentõs számú autoimmun beteg széruma is pozitív reakciót adott, noha glutén szenzitív enteropathiára utaló tüneteket e betegeknél nem észleltünk, és az endomysium antitest vizsgálat valamennyiüknél negatív eredménnyel zárult.

Következtetések. A TGc ELISA teszt alkalmas a glutén szenzitív enteropathia egyszerû és noninvazív diagnózisára, szûrésére és a betegek kontroll vizsgálatára. A humán TGc ELISA valamivel jobb eredményeket mutatott, mint a tengerimalac TGc ELISA, és egészséges kontrollokkal összehasonlítva ugyanolyan specifikusnak és szenzitívnek bizonyult, mint az endomysium antitest teszt. Azonban TGc ELISA pozitivitás más betegségekben is elõfordulhat, így pl. egyes autoimmun betegségek fennállása esetén önmagában nem diagnosztikus értékû.

1. SUMMARY

Role of tissue (type 2) transglutaminase in the pathogenesis and diagnosis of coeliac disease and dermatitis herpetiformis

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Basics of Human Molecular Genetics and Genetic Diagnostics
Budapest, 2002.

Introduction. Tissue (type 2) transglutaminase (TGc) has been identified as the target autoantigen of circulating, anti-endomysial IgA antibodies in coeliac disease (CD) and dermatitis herpetiformis (DH). An ELISA test based on the guinea pig TGc had been developed to detect these antibodies. To confirm the significance of this method in the diagnosis of CD and DH, we developed and validated an ELISA based on the human recombinant antigen and compared it to existing serological tests for CD and DH (guinea pig TGc ELISA and endomysium antibody test). Furthermore, to test the specificity of our ELISA and discover a possible wider autoantigenic role of TGc, also sera from patients with other disorders were also analysed.

Methods. Serum samples from healthy controls, patients with CD and DH, as well as sera from patients with other autoimmune or gastrointestinal disorders were investigated. ELISA assays for IgA antibodies against TGc using calcium-activated guinea pig liver and human recombinant TGc were performed. The sera were also tested for IgA endomysium antibodies using indirect immunofluorescence.

Results. The human TGc was expressed and purified as an active enzyme. When compared to healthy controls, the specificity and sensitivity were high for the diagnosis of untreated CD and DH in both the guinea pig and the human TGc ELISA systems. However, a considerable number of sera from patients with autoimmune disorders also showed positivity, though no association of these conditions with gluten sensitive enteropathy was apparent.

Conclusions. The TGc ELISA is suitable for use as a simple, noninvasive method for diagnosis, screening and follow-up of GSD. The human TGc ELISA was found to be somewhat superior to the guinea pig TGc ELISA, and as specific and sensitive as the endomysium antibody test when compared to healthy individuals. However, TGc ELISA positivity also occurred in patients with other disorders. Thus the presence of such antibodies should not be taken alone as a basis for the diagnosis of gluten-sensitive enteropathy.

2. INTRODUCTION

2.1. Definition and history of coeliac disease and dermatitis herpetiformis, the two forms of gluten-sensitive disease

Coeliac disease (CD; also known as nontropical sprue, gluten-sensitive enteropathy, or coeliac sprue) is a common chronic small bowel mucosal disorder associated with a persistent intolerance to gluten as well as concomitant immune and autoimmune (AI) phenomena (283, 182). Changes in CD are, however, not confined to the small intestine (2.4.1). Thus today a few scientists believe that it is primarily a systemic AI disease with frequent intestinal and infrequent (rarely diagnosed?) extraintestinal manifestations. In a few individuals, CD is associated with dermatitis herpetiformis (DH; also known as Duhring's disease or Duhring-Brocq's disease), a bullous, AI skin disease. It is characterised by polymorphic eruptions preferentially located over the extensor surfaces of the major joints with underlying granular IgA deposits occurring in the papillary dermis (96). As CD and DH share a very similar jejunal pathology, identical genetic background, similar pathomechanism, as well as common diagnostic analysis and dietary possibilities for therapy (96), the term 'gluten-sensitive disease' (GSD) for these conditions will be applied. For description of enteropathy in these two illnesses, the term 'gluten-sensitive enteropathy' (GSE) will be used.

The first report resembling CD originates from Aretaeus from Cappadochia in the II. century A.D. (266). Samuel Gee produced a classical description of CD over a century ago (102). However, he beleived it was a disease of the coecum and hence its name. The term 'sprue' derives from the Flemish word 'sprouw' meaning 'aphthous lesion' (140). An effective treatment for CD (with a diet containing exclusively meat and bananas or milk, fruits and vegetables) based on the incorrect belief that it was an intolerance to starch was described in the 1920s (114). However, it was not until 1932 that the Dutch paediatrician, Dicke, discovered its association with the consumption of certain cereals, later identifying gluten as the causative factor (68). Later studies showed the subtotal villous atrophy characteristic of CD (237) resolved following withdrawal of gluten from the diet (12). The small intestinal pathology showed that CD is identical condition both in children and in adults (234). Today it is known that both CD and DH are evoked and maintained by an abnormal small intestinal mucosa associated with a persistent intolerance to gluten, the adhesive mass of water-insoluble proteins of wheat, and other related cereal proteins present in rye, barley and probably oats, but not in rice and maize (68, 289). Removal of these from the diet leads to a full clinical and histological remission. Beside the immune response against cereal proteins, AI events play a significant role in the pathomechanism. Three types of tissue autoantibodies have been described in GSD. Seah et al., 1971, detected tissue autoantibodies (IgG, IgM, and in a few cases IgA) in both CD and DH by indirect immunofluoresence using rat tissues, as well as human thyroid gland and jejunum. These were called 'reticulin' antibodies (Abs) as their staining pattern resembled that seen by silver staining of reticular fibres (248, 249). In 1983, Chorzelski et al. reported the association between GSD and circulating IgA anti-endomysial Abs (EMA; 48). Kárpáti et al. described in 1986 the existence of specific IgA anti-jejunal Abs by indirect immunofluorescence (152), and as-

sumed them to be identical to the anti-endomysial and anti-reticulin Abs (146). Kárpáti et al. also demonstrated the ultrastructural localisation of IgA Abs in the endomysium (148). However, the target molecule of these autoantibodies remained unknown until 1997 when the dominant autoantigen of CD was identified by Dieterich et al. as tissue (type 2) transglutaminase (TGc; 69). This was subsequently demonstrated to be the common tissue autoantigen of both reticulin, endomysial, and jejunal Abs (163).

DH is a rare skin disease, first described by Louis Duhring in 1884 (76) and initially beleived to be a purely dermatological disorder. It was grouped with the various types of aquired epidermolysis bullosa, pemphigus, pemphigoid, and linear IgA dermatosis, which were referred to collectively as the 'bullous skin diseases'. A clear distinction between these disorders could be made upon the difference in the layer of blister formation (51) and the response to sulphonamides (58). Marks et al., 1966, discovered small intestinal mucosal lesions in DH (197) which led to the showing of the pathogenic role of gluten (93, 94, 252). Direct immunofluorescent staining of jejunal mucosa of DH patients showed the presence of immunoglobulins (IgG and IgA) in the basement membrane zone (Dick et al., 67), but it was not until 1988 that Kárpáti et al. described the specificity of IgA (147, 150). In 1969, van der Meer demonstrated that IgA was deposited in the papillary dermis of DH patients showing granular pattern (276), this was later described by Kárpáti et al. at the ultrastructural level (149). These deposits are generally considered immune complexes as sera from patients with DH do not stain normal human skin (67). Attempts to elute IgA Abs from the skin of DH patients to show their specificity, however, have failed (79). Recently, the autoantigen in the immune complexes of the papillary dermis of DH patients has been identified by our research group to be the epidermal (type 3) transglutaminase (TGe; 239).

2.2. Epidemiology

In contrast to the similar pathomechanism, the epidemiology of CD and DH is distinct. There is a fundamental difference in the prevalence: whereas DH is rare, CD is a common condition. In addition, there are also some minor differences in other epidemiological features which will be discussed later in this section. Neither appears in individuals on a gluten-free diet (GFD), and both usually affect the white population of countries where wheat, rye, and/or barley are important nutrients, i.e. Europe, Western and Northern Asia, parts of America and Australia (56). GSD is uncommon in Africa, as well as Eastern and Southern Asia. Black persons usually do not suffer from GSD even if they live in a European country and take gluten regularly (56). Among Asian races, GSD can occur, (mainly in Arabic countries and India), but is extremely rare (56). As race rather than emigration is important in the occurrence of the disease it is evident that genetic predisposition is one of the major aetiologic factors.

Epidemiological results are often difficult to evaluate (16). The definition of CD varies and it is now evident that the spectrum of pathological findings of the jejunal mucosa is somewhat wider than those defined in the ESPGAN criteria (villous atrophy, crypt hyperplasia, and increased number of intraepithelial lymphocytes). High counts of intraepithelial lymphocytes alone, without other abnormalities, may be a non-specific sign of GSE, and are often seen in patients on an incomplete GFD (16, 283). The original ESPGAN criteria (203) require two additional biopsy samples after removal and

reintroduction of gluten into the diet, the revised criteria require the third biopsy only where the diagnosis is uncertain (283). These criteria, although widely accepted as being absolutely correct, are often not fulfilled in practice. A single biopsy showing typical changes together with positive serological findings is often taken as sufficient for the diagnosis.

As well as the differences in criteria set for GSD diagnosis, the large variations between study results in areas of the same country or neighbouring countries might be due to genetic, cultural, or alimentary diversity. In addition, there are also frequency variations detectable in the same area for different periods of sampling.

2.2.1. Epidemiology of CD

Due to the difficulty in dating the onset of CD (see below), it is more informative to calculate the prevalence or the cumulative incidence of the disease than the incidence itself. The frequency data changed dramatically in the last decade. About 15-20 years ago, the reported cumulative incidence rate of coeliac disease in Europe was widely different ranging between 1:400-1:12800 births, (Sweden and Greece, respectively), a 32-fold difference between the highest and lowest rates with an average rate of 1:1000 (108). This difference might be due to the diverse genetic backgrounds, the differing amounts of gluten consumed, or the time point of first gluten exposure (24, 167, 284). In Hungary, the cumulative incidence rate between 1970 and 1985 of children with CD was 1:3941 (27). In 1987, 556 children with manifest CD (proven with three biopsies) were enregistered in Hungary (166). In a region of Hungary, the prevalence reached 1:650 (260).

With the development of sensitive screening tests for CD allowing detection of silent or atypical CD, the reported prevalence rates reached 1:100-1:300 in many European countries (108, 161). In Hungary, a recent study showed a prevalence rate of 1:85 among children between 3-6 years (161).

The largest, multicentre and nationwide study was performed during 1993-95 in Italy on 17201 students aged 6-15 years. Here, the anti-gliadin Ab (AGA) screening test was used as the first step, followed up by EMA testing (44). An unexpectedly high prevalence (1:184) was found, and most significantly the ratio of known to undiagnosed CD cases was 1:7. Italian epidemiologists designated this situation as the ‘coeliac iceberg’ (Fig. 1).

CD can manifest at any age but the majority of patients having symptomatic disease are diagnosed in the early childhood (mainly during the first two years of life, usually before 6 years of age) (108, 182 and references therein). In the last decades, a trend of increased number of patients diagnosed with CD at the age of 10 or later could be observed (108). Either CD is delaying its appearance to later ages over Europe, or the screening tests allow the diagnosis of adults with atypical, silent or latent disease. In Hungary, also an elevation of age at diagnosis could be observed. In 1975-77, the mean age at diagnosis was approximately 1.5 years, whereas in 1987-89 it reached 5 years (27).

The sex frequency is nearly equal in early childhood while in adults the female:male ratio is 2:1 (182, 128). Speculatively, CD may be more frequently undiagnosed in males, possibly because of pregnancy precipitating clinical disease (258). In Hungary, between 1963-1987, the female:male ratio of registered paediatric CD patients was 1.46 (445 girls and 304 boys) (Kósnai I, personal communication). In and near the capital

(Budapest and Pest county), the ratio was somewhat higher, 1.57 (girls:boys=137:87) (Kósnai I, personal communication).

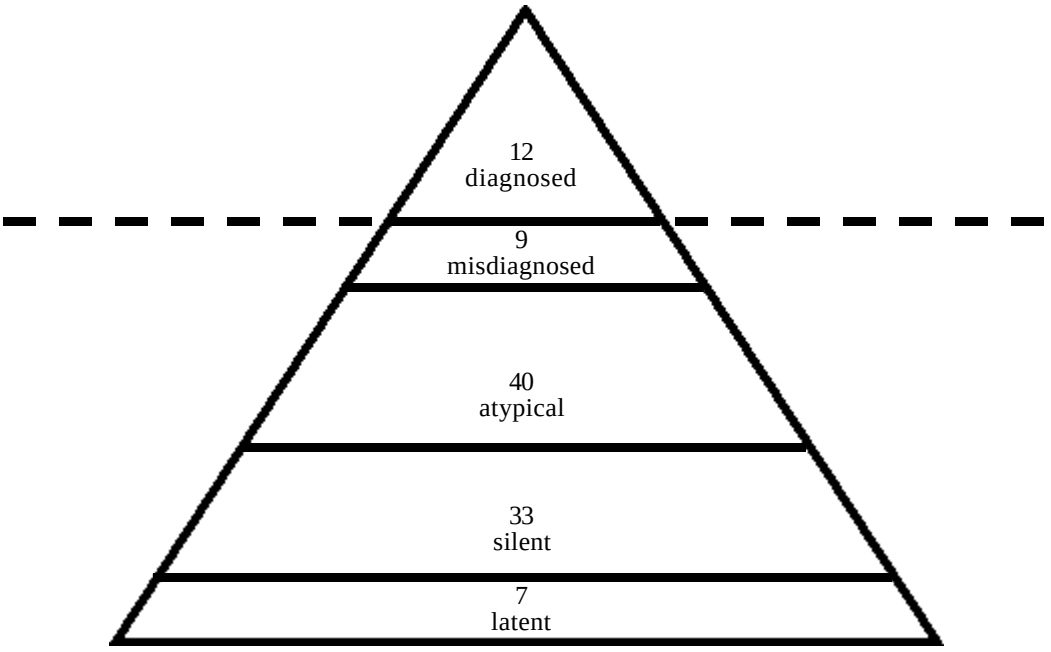

Fig. 1. The coeliac iceberg in Italy (44). Among the 17201 students examined in the study, 101 had CD but only 12 had been clinically diagnosed constituting the 'emerging part of the iceberg'. The other 89 cases were found by serological screening. Nine cases ('misdiagnosis group') had received a clinical diagnosis of gluten intolerance during infancy based on malabsorption symptoms. They had been treated with a GFD for a period of time and then a normal diet had been reintroduced. Patients with atypical CD had symptoms and signs which were not attributed to CD, e.g. monosymptomatic cases, extraintestinal, or unspecific symptoms. The latter two groups build a considerable number of patients with CD where in spite of the presence of clinical symptoms the disease is not diagnosed. Patients labelled with 'silent' CD are practically symptomfree, but a small intestinal histology (and serological tests) can show overt GSE. Patients having latent CD show apparently normal small intestinal architecture when examined with traditional histological methods, but they show positivity with other specific tests (positive EMA test on monkey oesophagus, increased ã/ä+ intraepithelial T-lymphocyte counts, and/or jejunal IgA deposits).

2.2.2. Epidemiology of DH

There have been only a few studies on the frequency of DH. Like CD, it is most common in Northern Europe. Prevalences for DH per 100,000 habitants range between 19.6-39.2 in Sweden (204, 206, 49), and are 11.4 and 11.5 in Finland and Edinburgh, respectively (231, 101). The incidence rate of DH in Sweden and Finland has been described as 1.05-1.45 and 1.3 among 100,000 people, respectively (204, 206, 49, 231). The frequency of DH in Hungary has not been investigated.

In contrast to CD, DH most commonly manifests in late adolescence and early adult life (15-40 years) (204, 206, 49, 231, 101). However, it may also present in infants and very old people. In most reported cases, DH is rare in childhood except for two countries, Italy and Hungary (96, 165, 230). In contrast to CD, the age at the manifestation of DH is not related to the amounts or the time point of first introduction of gluten (167, 230). Being a rare and very polymorphic disease of remitting-relapsing nature often presenting with symptoms other than blisters, DH might be underdiagnosed in certain areas, but the difference in age cannot be contributed to diagnostic unawareness of pediatric dermatologists in the Northern European countries.

Overall, the male:female ratio in DH is 3:2, but females predominate under 20 years of age (male:female=2:3; Ref. 96). In Hungary, the sex ratio of DH children is approximately 1:2 (male:female; Refs. 145, 167).

2.3. Pathophysiology

The multifactorial causes of GSD are discussed below.

2.3.1. Genetics of GSD

GSD is a condition in which the ingestion of gluten is not tolerated. However, the disorder occurs only in a susceptible population showing genetic factors are important. McDonald et al. suggested that CD reflected an autosomal dominant inheritance with incomplete penetrance (201). However, later studies suggested a purely recessive inheritance (111). It is now known that both HLA and non-HLA genes together with gluten and presumably additional environmental agents are involved in disease development. Diseases where particular HLA allels are overrepresented (e.g. type I diabetes mellitus and rheumatoid arthritis) are usually multifactorial and of a chronic nature (268). However, CD is unique in being strictly dependent on a well characterised environmental factor, the gluten ingestion.

Monozygotic twins with CD show a high concordance rate (70%) again suggesting strong genetic aetiology, further a high prevalence rate among first degree relatives of CD patients (10%) and among HLA-identical siblings (40%) also supports genetic influence (for review see 255, 72, and references therein). However, even though the overall importance of non-HLA genes appears to be greater than that of HLA genes, attempts to map predisposing genes by linkage analysis have failed to find frequently and obviously involved candidates. Recently, certain chromosome regions were suggested to be involved in the genetic difference between CD and DH (124). The cytotoxic T-lymphocyte associated gene 4 (CTLA-4, see Refs. 73 and 255) in the 2q33 chromosome region was suggested as an important non-HLA gene. However, investigations in more than 1000 affected families showed that other genes within this region (CD28 and the inducible costimulator ICOS, both being involved in the regulation of T-cell response) might be better candidates (Clerget-Darpoux F et al., oral communication). The strong linkage disequilibrium between these genes makes it difficult to find any primary susceptibility gene. However, there is evidence that at least one of the non-HLA risk factors is located in the 5q31-33 chromosome region, but the responsible gene(s) could not yet be identified. It is likely that each non-HLA gene has only minor

influence in altering the prediposition to GSD, which could explain the difficulty in delineating the genetic components.

2.3.1.1.The HLA system in general

The HLA system (reviewed in 36) consists of a complex gene series at several closely linked loci collectively called the 'major histocompatibility complex' (MHC). It is one of the most polymorphic genetic systems in mammals. HLA genes play important role in the immune system, including the immunopathology of certain diseases, and in allotransplantation rejection reactions. These genes are located on the short arm of chromosome 6 in a relatively short (3 cM, ~3400 kb) region together with some genes unrelated to known immunological functions, whereas not all genes involved in the regulation of immune response are located in this region (36). The glycoproteins they encode are expressed codominantly.

Three classes of gene products have been identified (36). Class I molecules, expressed on virtually all nucleated cell surfaces, are the products of three major reduplicated loci: HLA-A, -B, and -C. (Genes of minor significance are also present called HLA-E, -F, and -G). Class II molecules are restricted in expression to B-lymphocytes, some monocytes-macrophages, dendritic cells, skin Langerhans cells, and activated T-lymphocytes. They consist of two polypeptide chains (á and â) of unequal length and are the product of HLA-D genes which are subdivided into the subclasses termed DR, DQ, DP, and the less important DM, and DO. Class III molecules are the C4, C2 and Bf components of the complement system. Class I and II molecules form complexes with immunogenic peptides and are recognised by antigen-specific T-lymphocytes. Antigen-specificity of T-lymphocytes is achieved during development in the thymus. Most AI inflammatory diseases are thought associated with certain HLA combinations rather than caused by the HLA haplotype.

A WHO committee periodically assigned universally accepted designations to individual alleles of each HLA locus according to serological characterisation (e.g. HLA-A1, -B5, -Cw1, -Dw1 etc.). The numbers did not indicate the order of genes or their distance from each other as they had been identified before exact localisation occurred. Provisional alleles were labelled with 'w'. Recently, based on DNA sequence of these genes, the nomenclature was changed to reflect the extreme polymorphism not clearly distinguishable by serological technique. For instance, HLA-DR1 is written as DRB1*0101. DRB1 indicates the â chain of the DR locus, *0101 shows that it is antigen 1, first variant (36).

2.3.1.2.The role of HLA system in GSD

Both CD and DH occur in the same genetic background. Serological studies showed that more than 90% of GSD patients carry either the DR3-DQ2 haplotype (and are homozygous) or are DR5-DQ7/DR7-DQ2 heterozygous (255 and references therein). The products of these two genes interact with each other forming a class II heterodimeric molecule. Sequence studies showed that the HLA-DQA1*0501 and the HLA-DQB1*0201 alleles occur in the DR3-DQ2 haplotype. These two alleles can also be found, however, in the combined DR5-DQ7/DR7-DQ2 heterozygous haplotypes. The DR5-DQ7 haplotype carries the DQA1*0501 and DQB1*0301 alleles, the DR7-DQ2 haplotype carries the DQA1*0201 and DQB1*0202 alleles (DQB1 *0202 and *0201 being almost identical). However, about 2-10% of GSD patients do not carry the above

haplotypes. Here CD is most probably associated with the DR4-DQ8 haplotype carrying the DQA1*0301 and DQB1*0302 alleles (255).

In summary, susceptibility to GSD appears primarily associated with the alleles HLA-DQA1*0501, DQB1*02 (encoding the HLA class II heterodimer molecule HLA-DQ2), and to a lesser extent with the HLA-DQA1*03, DQB1*0302 genes (encoding the DQ8 molecule) (255).

2.3.2. Gluten, the harmful protein complex

Gluten is a protein complex forming the adhesive mass of cereals. Its adhesive feature contributes to the stickiness of wet flour. Physiologically, gluten is a storage protein providing nitrogen source for the seed embryo during germination. Gluten can be subdivided into two major components upon solubility: prolamines and glutenins (289). The former proteins are more soluble in 70% ethanol. The extraction of these components from the flour does not lead to uncontaminated fractions. Prolamins are the major toxic constituents, with glutenins beleived to be far less harmful (65, 289).

Prolamines of wheat (called gliadins), rye (secalins), and barley (hordeins) contain particularly high amounts of glutamine (> 32 mol%), proline (> 15 mol%), and hydrophobic amino acids (approx. 19%, mainly phenylalanine) (57, 83, 289). Phylogenetically, wheat is most closely related to rye and barley, and though the prolamines of oats (avenins) are also composed of high amounts of glutamine, they differ in their low proline content. Based on *in vitro* and *in vivo* observations together with the biochemical structural differences, pure oats is believed to be non-toxic, or at least far less harmful than its highly toxic phylogenetic relatives (135, 136, 137, 223). Oats grown elsewhere than in Northern countries (e.g. Central Europe), is often contaminated with other toxic cereals, thus in these countries oats cannot be recommended as nutrient for GSD patients. Prolamines of rice (oryzas), maize (zeins), and millet (pennisetums) are proven to be innocuous.

Gliadins are subdivided into á, â, ã, and ù gliadins, displaying apparent molecular weights on electrophoresis between 20-75 kD (289). *N*-terminal structural studies have shown â gliadins to be part of á gliadins (23). Only the primary structure of gliadins is important in the pathogenesis of GSD, because heat- or proteinase-treated polypeptides (with the exception of treatment with papain or heat far above 100º C) do not loose their toxic effect (289). á gliadins are the most toxic components, but all forms can be pathogenic (127). All three types of gliadin consist of more than 100 different protein variants with more than 90% of homology (289). They all contain high amounts of repetitive oligopeptide sequences. Two tetrapeptides ('Pro-Ser-Gln-Gln' and 'Gln-Gln-Gln-Pro') occur in all toxic proteins and appear absent from harmless forms (289). Gluten peptides (both gliadins and glutenins) become more toxic after deamidation of certain glutamine residues by a transglutaminase enzyme (see section 2.3.4).

2.3.3. Transglutaminases

Beside genetic predisposition and gluten ingestion, also TGc (and TGe) play a role in the pathogenesis of GSD.

2.3.3.1. History of transglutaminases

Cross-linking of fibrin polymers was first reported by Barkan and Gaspar in 1923 (18). At that time, it was not clear why the solubility of fibrin in urea varied. Only in 1948 could Laki and Lóránd attribute the insolubility of fibrin to a Ca^{2+}-dependent pro-

tein termed 'fibrin-stabilising serum factor' (or 'Laki-Lóránd factor'; 176, 190, 191). This 'serum factor' (an enzyme) was subsequently purified (186). The protein was later termed 'blood coagulation factor XIII' upon the demonstration a patient with hemophilia due to its deficiency (74). In 1966, Lóránd et al. realised that the 'blood coagulation factor XIII' is an isoenzyme of the transglutaminase family (30).

Waelsch et al. (242) observed in 1957 the ability of a soluble liver protein fraction (containing TGc) to incorporate labelled amines (e.g. ^{14}C-cadaverine) into proteins in the presence of Ca^{2+}. The designation 'transglutaminase' was first used by Waelsch and co-workers, and later corrected by the Enzyme Commission (EC 2.3.2.13, transglutaminase = R-glutaminyl-peptide, amine-ã-glutamyl transferase).

Achyuthan and Greenberg showed in 1987 that TGc binds GTP resulting in inhibition of its activity (1), hinting at the reason why TGc later received a second name ($Gá_h$) when it was discovered as a G protein with a role in signal transduction (216).

In the skin, ã-glutamyl-å-lysine cross-links were found in hair protein extracts by Harding and Rogers in 1971 (117). The realisation that this TG was not identical to factor XIII or TGc (50), led to it being labelled 'epidermal' or 'hair follicle' TG (corresponding to TGe). Later the finding that both membrane-bound and soluble fractions contained TG activity (265, 181) suggested the presence of further epidermal TGs. The insoluble, 'keratinocyte-specific' (=TGk) TG was detected in cultured keratinocytes unlike the soluble 'epidermal, hair follicle' TGe. However, antibody studies suggested the expression of TGk, TGc, and TGe both in the hair follicle and epidermal keratinocytes (220, 235). This confusing situation led to the numbering of TG isoenzymes and their genes (220, 156) where 'TGM' denotes the gene and 'TG' the protein product both followed by an arabic number. TGM1/TG1, TGM2/TG2, and TGM3/TG3 were assigned to TGk, TGc, and TGe, respectively. The numbering did not alter the nomenclature of factor XIIIa and band 4.2, and enabled the classification of new members. As this nomenclature is still not generally accepted, traditional names will be used below.

Subsequently other TGs have been discoved either through protein isolation or by sequence homology. Hence TGp (TG4) was isolated from prostate adenocarcinoma cells (31), and more recently, Aeschlimann and co-workers (3, 113) have identified three new family members: TGx (TG5), TGy (TG6), and TGz (TG7). The erythrocyte membrane protein band 4.2 was also found to belong to the family. It has over 30% similarity to certain TG isoenzymes; however, a cysteine to alanine substitution within its active site renders it catalytically inactive (164). Thus nine distinct TG isoenzymes are known in man today.

2.3.3.2.Transglutaminases in general

TGs are universal enzymes present in both pro- and eukaryotic cells and tissues including fungi and plants. They play very different physiological roles. Every cell type seems to express at least one isoenzyme. In man they may be expressed as both extra- and intracellular enzymes (Table I on page 19). Their primary structure has already been determined leading to their recombinant expression (with exception of the ones very recently discovered). Moreover, the three-dimensional structure of a number of isoenzymes (e.g. factor XIIIa and TGc; see Refs. 292, 184) has been described allowing a greater understanding of their catalytic mechanism. The transglutaminase induced posttranslational modification reaction (EC 2.3.2.13; also referred to as the R-glutaminyl-peptide, amine-ã-glutamyl transferase reaction), leads physiologically to the Ca^{2+}-dependent cross-linking of a specific glutamine residue in the substrate protein to

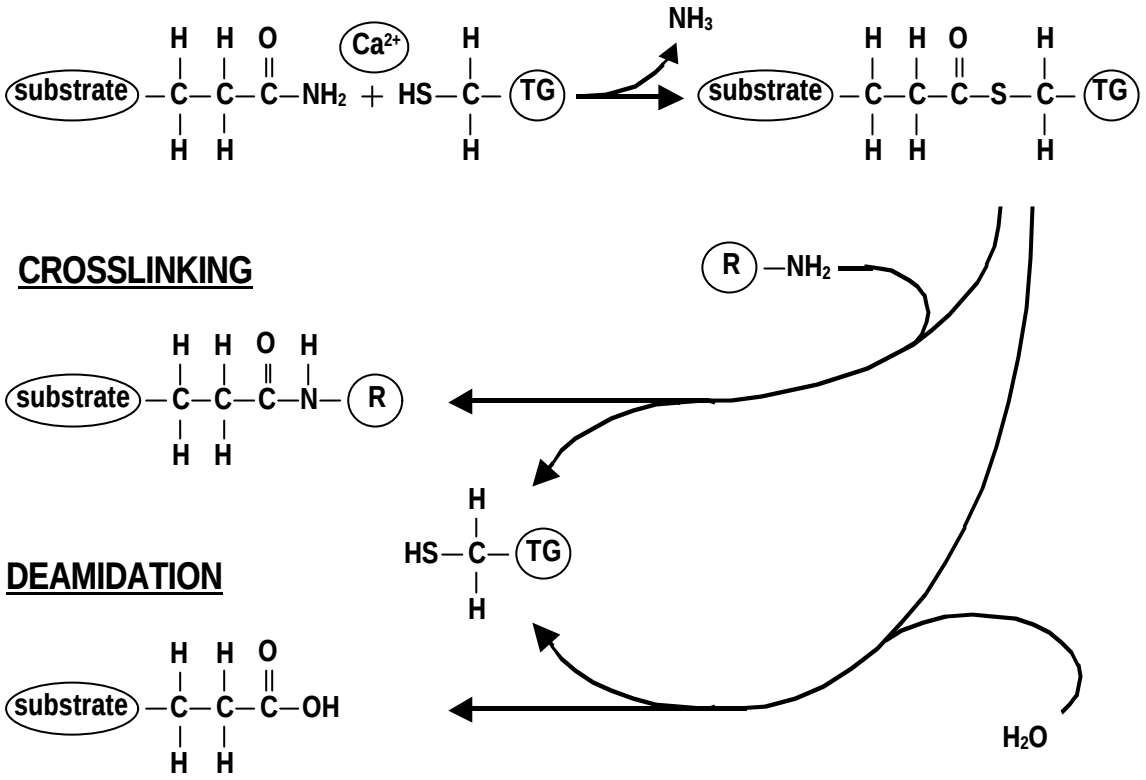

Fig. 2. Simplified scheme of enzymatic reactions catalysed by TGs. The TG reaction occurs in two steps (see references in the text). Initially, the substrate binds with the ã-amino group on a glutamine side-chain to the catalytic-site thyol (cysteine) of the TG enzyme. This step can only occur in the presence of Ca^{2+}-ions, and results in the release of NH_3 and formation of an acyl-TG intermediate. The second step is the reaction between the acyl group of the substrate and an available acyl acceptor primary amine (labelled with 'R-NH_2' in the figure). The primary amine can be either a protein-bound lysine resulting in the formation of a ã-glutamyl-å-lysine isopeptide bond (within the same or between different polypeptide chains), or a naturally occurring polyamine like putrescine or spermidine. However, in the absence of amines, a reaction with H_2O can occur at a significantly slower rate, which will produce deamidation of the substrate creating a new glutamate residue. In case of excess amounts of enzyme but limited amounts of substrates, certain TG isoenzymes can serve as their own substrate, this phenomenon is called 'autocatalytic cross-linking'.

a primary amine which can be either a polyamine (e.g. histamine, serotonin; but also some drugs such as isoniazid) or a protein-bound lysine, the latter resulting in the formation of a ã-glutamyl-å-lysine isopeptide bond (88, 89, 189, 5, Fig. 2). This reaction usually creates a covalently connected polymer of proteins.

The TG reaction occurs in two steps (88, 89), the first step being the binding of a glutamine side-chain to the catalytic-site of the TG (Fig. 2). The second step is usually the reaction between the substrate and an available primary amine, but in the absence of amines, a reaction with H_2O can also occur (at a significantly slower rate at pH 7.4, but predominating at lower pH values), which results in deamidation of the substrate (86, Fig. 2). TGs can also catalyse the hydrolysis of certain esters by reaction with H_2O or

alcohols (86, 89). This catalytic mechanism and the peptide sequence near the active-site cysteine resembles thiol proteases. Energetically, the TG reaction causes only very low change in the free energy, the driving force for the reaction is supplied by the release of NH_3 and its subsequent protonation.

Theoretically, the TG reaction is reversible, but the immediate protonation of NH_3 prevents this. Further the cross-linking almost certainly reduces accessibility so it is unlikely that the isopeptide bond can be cleaved without prior degradation of the protein. Hence under physiological circumstances, the TG reaction is practically irreversible. After lysosomal proteolysis of cross-linked molecules, the isopeptide bond of the arising dipeptides can be splitted by the enzyme ã-glutamylamine cyclotransferase which produces lysine and pyroglutamic acid (84).

In general, TGs are highly specific for their glutamine containing substrates, but they have almost no specificity for the amine containing substrates. Only relatively few glutamine donor substrates are known. Which protein can be a physiological substrate strongly depends on the function of the relevant TG isoenzyme, as yet no general consensus sequence for substrate usage of distinct isoenzymes has been described. It is postulated that this specificity depends rather on the conformation than the sequence of the substrates. Interestingly, FXIIIa, TGc, and TGx have been shown to act as their own substrates resulting in the autocatalytic cross-linking of the enzymes into multiples of high molecular weight (19, 34, Sárdy et al., unpublished observation). Further, TGc is also able to incorporate primary amines (e.g. histamine or putrescine) into itself (189).

TG production and activity is strictly regulated in mammals although little is known about the regulatory mechanisms. Most isoenzymes are controlled uniformly by Ca^{2+}-ions (Table I), with a minimum Ca^{2+}-level of $1\text{-}5\times10^{-4}$ M (Ref. 189) required for activation (normally intracellular Ca^{2+}-concentration being 10^{-6}-10^{-7} M). In addition, some TGs (FXIII, TGk, and TGe) are also regulated by limited proteolysis (Table I).

Some of the TGs also act as GTPases and ATPases (Table I). This function is independent of the TG activity, but binding of GTP or ATP to the relevant isoenzyme can influence substrate binding to the catalytic core (1).

The most important features of TGs are summarised in Table I. The genes encoding for individual TGs are highly conserved across mammalian species (5), and the family members share a strong sequence conservation especially in their active sites (113, 5). Despite this they have rather distinct biological functions. The specialisation can be of so high degree that the absence of a particular TG isoenzyme cannot be replaced by another one. For instance mutations in TGk which result in lamellar ichthyosis cannot be compensated for by TGe, which is also involved in the cornified envelope formation (20, 129). Similarly, knockout experiments have shown TGe cannot be replaced by other TG subtypes (Milstone L et al., personal communication). The isoenzymes also differ in their own modifications (proteolytic activation, phosphorylation, fatty acylation), which regulate their enzymatic activity and subcellular localisation (6).

Not only the biological function but also the pathological roles of TG isoenzymes differ. TGc has been identified as the major autoantigen of disease-specific circulating IgA Abs in CD and DH (69, 70), and TGe has been implicated in the pathogenesis of DH (239). The importance of TGc is better understood and will be described below.

Table I. Comparison of features of all known transglutaminase isoenzymes[5,6,3,11,33,45,92,106,110,123,122,154,155,156,213,219,233,245,250,257,267, and references therein]

TG isoenzyme	**FXIIIa**	**TGk**	**TGc**	**TGe**	**TGp**	**TGx**	**TGy**	**TGz**	**EB4.2**
Gene name	F13A1	TGM1	TGM2	TGM3	TGM4	TGM5	TGM6	TGM7	EPB42
Chromosomal localization	6p24-25	14q11.2	20q11-12	20q11	3p21-22	15q15.2	20q11	15q15.2	15q15.2
Gene size	~160kb	~14kb	~37kb	~43kb	~35kb	~35kb	~45kb	~26kb	~20kb
Number of amino acids without the 1. methionine	731	816	686	692	683	719	706	709	690
Molecular weight (kDa)	~83	~106	~77	~77	~77	~81	~79	~80	~72
Primary molecular features	Exists as zymogen consisting of 2 catalytic a subunits and 2 noncatalytic b subunits	Exists as zymogen, trimeric after proteolytic activation (67/33/10 kDa), both cytosolic and membrane-associated	Monomeric, a shorter splicing variant is beleived to exist having uncontrolled cross-linking activity	Exists as zymogen, the ~50kDa proteolytic fragment has cross-linking activity	Monomeric	Monomeric, 3 other splicing variants	?	?	Monomeric
Cross-linking activity	Yes	Yes	Yes	Yes	Yes	Yes	?	Yes?	No
GTP effect on cross-linking activity	None	None	Inhibition	Inhibition	Inhibition	Inhibition	?	?	–
Ca^{2+}-activation	Yes	Yes	Yes	Yes	Yes	Yes	?	Yes?	–
Proteolytic activation	Yes	Yes	No	Yes	No	No	?	?	–
Presence in the skin	Yes	Yes	Yes	Yes	No	Yes	No?*	Yes	Yes
Presence in other cells, tissues or organs	Blood plasma, platelets, monocytes-macrophages, hepatocytes, chondrocytes, placenta.	Epidermal and hair follicle keratinocytes, epithelial cells in stratified squamous epithelia, endometrium, major ducts of pancreas and mammary glands	Widespread	Mouse: brain, stomach, spleen, small intestine, esophagus, testis, skeletal muscle; human: kidney and lung.	Prostate only (some is expressed also in salivary glands)	Widespread	?	Widespread	RBCs, platelets, fetal liver and kidney, adult brain, adult kidney?
Consequence of deficiency in human	Impaired blood coagulation, spontaneous abortions	Lamellar ichthyosis	Maturity-onset diabetes of the young?*	Unknown (no implantation due to early embryonal lethality*)	Unknown	Unknown	Unknown	Unknown	Enhanced RBC fragility (spherocytosis, hemolytic anemia)

* Personal communications.

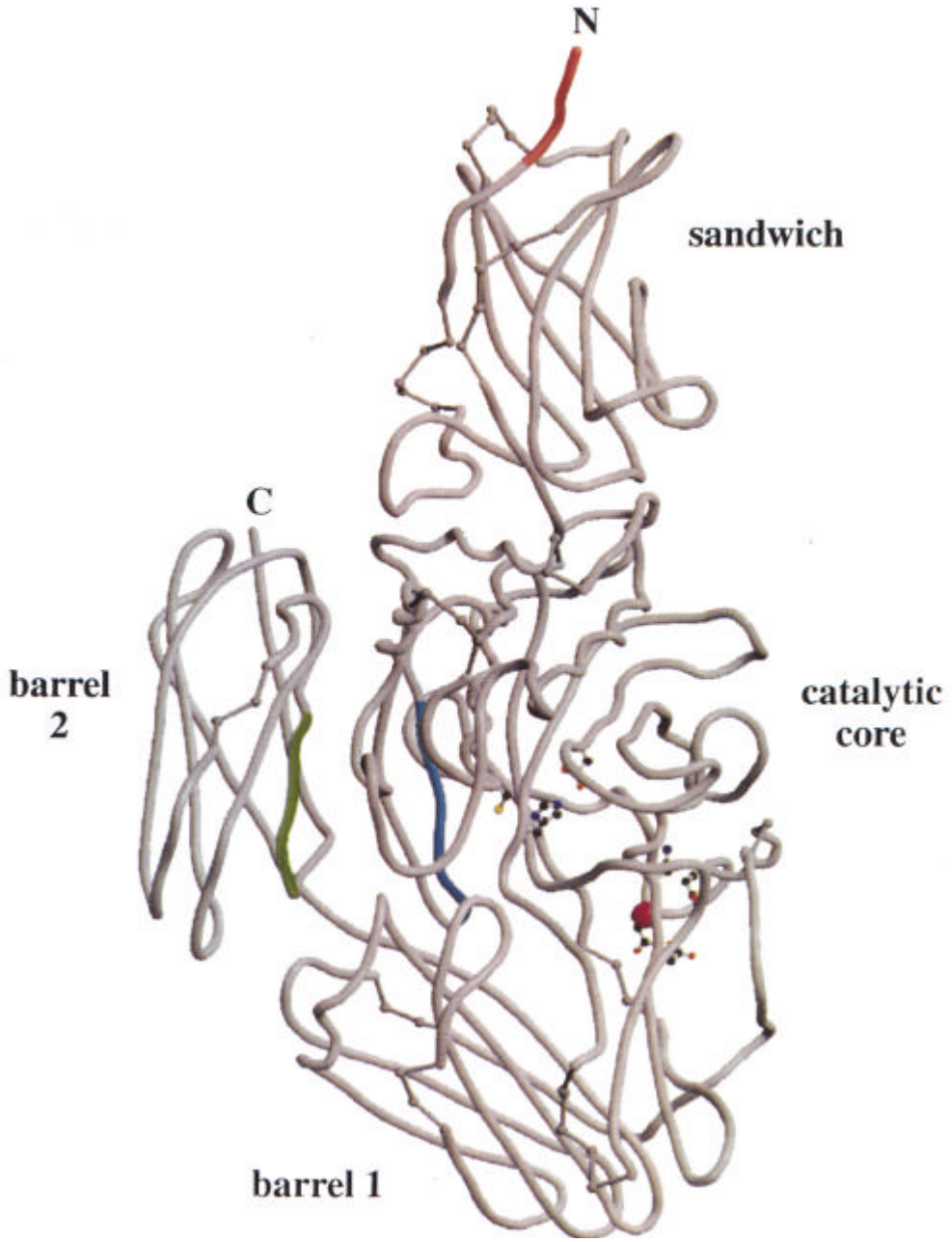

Fig. 3. Three-dimensional structure of TGc (132). A computer-generated structure of TGc was constructed based on the X-ray crystallographic structure of factor XIII a-subunit (132). Residues forming the active site of TGc (Cys^{277}, His^{335}, Asp^{358}) and those (Asp^{399}, Ser^{418}, Glu^{446}, Glu^{451}) involved in complexation of a Ca^{2+}-ion (red sphere) are represented as ball-and-stick side chain groups. Proposed (132) binding sites for GTP (blue; Gly^{165}-Lys^{173}), fibronectin (red; acetyl-Ala^{2}-Glu^{8}), and phospholipase C (green; Val^{665}-Lys^{672}) are high-lighted in colour.

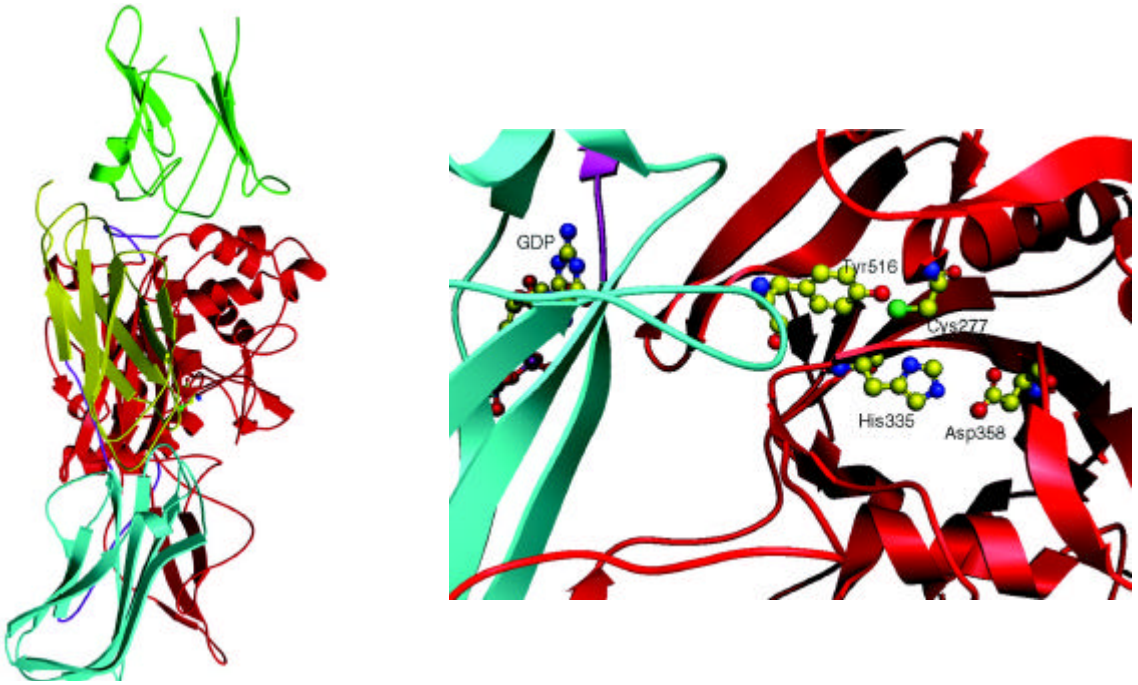

Fig. 4. Three-dimensional structure of human TGc (modified from 184). *Left*, overall structure. The ribbons of the â-sandwich domain, catalytic core domain, the first and second â-barrel domains are shown in green, red, cyan, and yellow, respectively. The loops connecting the first â-barrel to the catalytic core and the second â-barrel are drawn in purple. *Right*, the active site showing the catalytic triad relative to the GTP-binding site. Cys^{277}, His^{335}, Asp^{358}, Tyr^{516}, and GDP are shown in ball-and stick.

2.3.3.3.Features of TGc

TGc (also referred to as TG2, 'cellular TG', or 'tissue TG') is a multifunctional enzyme having several functions both in the intra- and extracellular space.

2.3.3.3.1. Enzymology, structure, and regulation of TGc

The human TGM2 gene localises to chromosome 20q11-12 (103), and its 13 exons span ~37 kb. The protein of 687 amino acids (together with the first methionine) has a calculated molecular mass of 77.3 kDa (103, 91). The active site involves three main residues (Fig. 3 and Fig. 4): Cys^{277}, His^{335}, Asp^{358} (104). This catalytic triad together with the majority of adjacent amino acids is extremely conserved in enzymatically active TG isoenzymes. After transcription of TGc in the free cytoplasmic space, it is N-terminally modified by removal of the first methionine residue and N-acetylation of the penultimate alanine residue (134). TGc contains no disulfide bonds, and is not glycosylated (133). In spite lacking the characteristic hydrophobic leader-sequence of a typical secreted protein (134), a proportion of TGc is found active in the extracellular space (2, 6, 8, 179, 272, 279, 162, 163). Its mode of release is still unknown. FXIIIa and TGp have also been found to function in the extracellular space (these TGs also lack a signal peptide).

The three-dimensional structure of recombinant human TGc has recently been determined (Fig. 4; Ref. 184), former models derived from that of FXIIIa (Fig. 3; Refs. 292, 132). Similarly to FXIIIa, it consists of an N-terminal â-sandwich domain [between amino acids (aa) 1-138], the catalytic core (aa 139-471), and two C-terminal bar-

rels (aa 472-584 and 585-687; see Fig. 3, Fig. 4, and Refs. 132, 184). Expression of just the catalytic domain results in a protein with no cross-linking activity, but a construct lacking the two barrels was shown to have (reduced) activity (132). The ATP/GTP binding site has been proposed to localise between aa 173-174, 476-483, and 580-583, the Ca^{2+}-binding site between aa 400-455, the fibronectin binding site between aa 1-7, and the phospholipase C binding site between aa 665-672 (132, 184).

The expression of human TGc is regulated differently depending on the cell type. It can be regulated by various cytokines, hormones, and drugs (reviewed in 180, 6 and 45). Cultured cells overexpressing TGc show morphological changes and enhanced resistance to proteolytic detachment indicating an increased adhesion; however, neither increased cross-linked products (105, 221) nor TGc activity (279) could be detected showing that the activity of TGc is independently regulated from gene expression.

The cross-linking function of TGc is allosterically activated by Ca^{2+}-ions and inhibited by GTP, GDP, and GMP, whereas it is not influenced by physiological concentrations of ATP or CTP (1, 21, 174, 175, 262). However, its GTPase and ATPase activity is independent of Ca^{2+}-ions, but depends on Mg^{2+}-ions, because Mg^{2+}-GTP and Mg^{2+}-ATP are the true substrates for the hydrolysis reaction (175). Further, the GTPase function is inhibited by the binding of ATP, ADP, and AMP (175). Divalent cations other than Ca^{2+} (and Sr^{2+}) usually do not activate TGc, whereas Fe^{2+}, Cu^{2+}, Zn^{2+}, and Hg^{2+} are strong inhibitors of the cross-linking activity (85). The intracellular modulation of TGc activity is also influenced by the Ca^{2+}-dependent protease calpain which effectively degrades TGc and is inhibited by GTP (294). Calreticulin inhibits both the TG and the GTPase activities (80). Certain molecules such as iodoacetamide and cystamine can irreversibly inhibit the cross-linking activity by alkylating the catalytic SH group (87). NO can regulate TGc by S-nitrosylation whose degree depends on the Ca^{2+}-concentration (202, 174). The situation is even more complex as TGc can also be activated allosterically by the membrane lipid sphingosylphosphocholine (173), and other unidentified factors might also participate in the regulation of enzyme activity.

2.3.3.3.2. Distribution, substrates and functions of TGc

TGc is expressed in virtually all cell types and tissues of the body suggesting essential role in their existence (4, 7, 267). Intracellularly, it is located in the cytosol and the nucleus, as well as associating with the cytoskeleton and cell membrane (6, 179). Its distribution is independent of the TG activity (6).

In terms of GSD it is important that TGc is expressed in the small intestine both in the epithelium and in the stroma. Jejunal biopsies of CD patients show increased TGc activity (29).

Despite extensive research on TGc, little is known about its real physiological functions. A number of both intra- and extracellular proteins have been shown to be substrates of TGc. Some examples are shown in Table II. Further, the significance of its deamidation reaction, GTPase, ATPase, and G-protein activity is still not understood.

TGc is implicated in physiological processes as diverse as extracellular matrix stabilisation in development and wound healing, hormone receptor signal transduction as a G-protein, receptor-mediated endocytosis, cellular adhesion, cornified envelope formation in the keratinocytes, and programmed cell death (for review see 6). However, its exact functions remain unclear. Indeed disruption of the TGM2 gene in the mouse does not lead to major phenotypic changes (64, 217) suggesting compensation by other family members. It is speculated that the insufficiency of TGc might lead to the rare disease

called maturity-onset diabetes of the young (MODY) (Melino G et al., oral communication), because TGc-nul mice show elevated blood sugar values. In addition, they develop anti-nuclear Abs after the first year of life, which may result in an SLE-like disease (Fésüs L et al., oral communication).

TGc has been implicated in the development of most major organs (reviewed in 6). In particular, TGc has been described as having an important role in cell adhesion and spreading mediated by its high affinity binding of cell surface-associated fibronectin as well as â1 and â3 integrins so acting as a coreceptor for fibronectin (8, 279, 272, 100, 69, 163). This function is independent of any cross-linking activity, but the association of TGc with the pericellular matrix of cells depends upon the N-terminal fibronectin binding domain of TGc (100). However, TG activity may be important in cross-linking (hemi-)desmosomal components, such as desmoplakin, with elements of cornified envelope and dermoepidermal anchoring filaments (78). TGc is also beleived to be important in the stabilisation of extracellular matrices such as the basement membrane and growth plate (2, 4, 6, 139). Furthermore, TGc has been associated with apoptosis (82), and it has been proposed that its intracellular activation by elevated intracellular Ca^{2+}-levels could lead to the irreversible assembly of a cross-linked protein scaffold in dead cells thus preventing the release of harmful intracellular components into the extracellular space. However, results from the TGc-/- mice suggest that all these functins are not TGc-specific (64, 217).

Table II. Substrates of TGc (selection from 6, 45, 157, 247, and 222).

Acetylcholine receptor
Actin
â-amyloid
Calreticulin
CD38 antigen
Collagens (e.g. type I, II, III, V, VII)
Crystallins
Dermatane sulfate proteoglycans
Elafin
Entactin
Fibrin and fibrinogen
Fibronectin
Fibrillin
GST P1-1
Heat shock proteins (e.g. Hsp23)
Histones (e.g. H1, H2A, H2B, H3, H4)
Huntingtin
Immunoglobulins (e.g. IgG)
Interleukin 2
Keratins
Lactate dehydrogenase
Lipocortin I
Lipoprotein A
Midkine
Myelin basic protein
Myosin
Nidogen
Osteonectin
Osteopontin
Phospholipase A_2
Plasminogen
Plasminogen activator inhibitor 2
Retinoblastoma protein (pRB)
ô-protein
TGF-â
Troponin
Tubulin
Vitronectin
Von Willebrand factor

In conjunction with FXIIIa, TGc is thought be significant in the stabilisation of the fibrin clot and the extracellular matrix after injury (109, 118). During healing, endothelial cells, macrophages, and skeletal muscle cells express increased amounts of TGc, and it has been shown active in the extracellular space within the wound (118).

A unique feature of TGc is its G-protein function. For historical reasons, it is also called the á-subunit of G_h ($Gá_h$). Together with $Gâ_h$, which has recently been suggested to be identical to calreticulin (80), TGc has been demonstrated to transmit the $á_1$-adrenergic receptor signal to phospholipase C-ä1 (216). However, transgenic mice overexpressing TGc in the heart showed no evidence for TGc acting as a G-protein-like

transducer but rather cardiac remodeling resulting in mild hypertrophy and diffuse interstitial fibrosis presumably due to TG activity (254).

In conclusion, while a body of evidence suggests that TGc is a multifunctional enzyme with roles in a number of metabolic processes, it appears that its functions are not specific and can be compensated for in its absence. Further studies are necessary to demonstrate unambigiously the functions of TGc.

2.3.3.3.3. Pathogenic role of TGc in diseases different from GSD

TGc has been implicated in the pathogenesis of several illnesses including immune or autoimmune (AI) disorders such as AI hepatitis, SLE, myasthenia gravis, haemolytic anaemia, bullous pemphigoid, Goodpasture syndrom, rheumatoid arthritis, amyotrophic lateralsclerosis, multiple sclerosis, and Crohn disease (242, 281, 222, 224, 157, 61), neurodegenerative diseases [Alzheimer's disease (251, 75, 138), Huntington's chorea (35), Parkinson's disease (138, 90, 157), progressive supranuclear palsy (7), dentatorubral-pallidoluysian atrophy (142, 131), spinobulbar muscular atrophy (280), various forms of spinocerebellar ataxias (142, 280)], malignancies (62, 282), human immunodeficiency virus infection (9), cataract formation (17, 188, 157), atherosclerosis (28, 288), and inclusion body myositis (46).

In the majority of these diseases, the action of TGc can be traced back to its cross-linking activity with cross-linked products beleived to behave as neoantigens in AI disorders. Proteins (amyloid peptides, ô protein or huntingtin) containing long polyglutamine regions may be cross-linked by transglutaminases and produce the cytosolic proteinaceous aggregates in neurodegenerative diseases. Also, enhanced cross-linking seems to contribute to cataract formation, atherosclerosis, and inclusion body myositis. In contrast, the role of TGc in metastasis process is more complex. Possibly the down-regulation of TGc as often found in metastatic tumours results in both reduced apoptosis of neoplastic cells and reduced adhesion to the extracellular matrix facilitating uncontrolled tumour growth and dissemination. The function of TGc in GSD appears to be different to that described in the diseases above and is detailed in the next section.

2.3.4. Pathomechanism of GSD

The manifest GSD with typical symptoms and signs is the result of culmination of four factors: genetic predisposition, gluten ingestion, small intestinal lesions, and eventual unmasking factors.

Despite the strong association with specific HLA haplotypes, genetic factors alone do not decide the clinical outcome of GSD. Monozygotic twins may exhibit any combination of manifest CD, DH or clinically silent GSD (96, 165) proving environmental factors are also significant. Although as stated above (2.3.1) several non-HLA genes are likely to be important in the pathogenesis of GSD, in practice all GSD patients share the same two HLA antigens: DQ2 (95% of patients) or DQ8 (almost 5%).

The main environmental factor in GSD is the ingestion of gluten in cereals. The amount of gluten consumed and the time point of first exposure may play a prominent role in CD manifestation (284). As detailed above (2.3.2), prolamines contain high amounts of glutamine, proline, and hydrophobic amino acids (57, 83, 289). These amino acids create several epitopes in the prolamin (e.g. gliadin) peptides, some are immunodominant. Indeed, T-cell clones carrying HLA-DQ2 or -DQ8 molecules and responsive to gliadin peptides can be isolated from the intestinal mucosa (192). Nevertheless,

there are only a few intestinal T-cells recognising native prolamin peptides as foreign antigens (192, 253). However, a special, peculiar feature of gluten is that the deamidation of certain glutamine residues in some polypeptides (both in prolamins and glutenins) can lead to major effects on their recognition as antigens with T-cells recognising certain deamidated polypeptides much more effectively than unmodified ones (192, 253, 207, 277; and reviewed in 255). In particularly, the majority of antigenic gliadin peptides are not recognised at all in their native form (14, 253, 207, 277). The explanation of this phenomenon is the characteristic property of DQ2 and DQ8 binding motifs that preferentially bind peptides with negative charges. Although gluten peptides are not rich in negatively charged residues, after deamidation creating glutamic acids from glutamines, gluten can become a rich source of DQ2 and DQ8 epitopes. Due to their high glutamine and proline content, prolamins are preferred substrates for TGc (29, 261). Recently, TGc substrate consensus sequences have been reported and shown present in gluten peptides (273). These confirm the important role of high proline content. While the deamidation reaction theoretically should not occur in the small intestinal mucosa where the pH is around 7.4 and high amounts of amine donors are present, T-cell lines isolated from small intestinal biopsy specimens and challenged with different gliadin preparations showed that coincubation of gliadin with the TGc inhibitor cystamine often reduced their responsiveness to deamidated gliadin. This suggests that the deamidation of gluten peptides may indeed be mediated in the mucosa *in vivo* by endogenous TGc (207, 208).

As TGc is localised directly under the jejunal villus epithelium (207, 163, 226) and its expression in biopsies of CD patients is elevated by 3.2-fold over that in healthy individuals (29), deamidation of gluten peptides may be more efficient in these patients. It is also possible that deamidation occurs on the apical surface of enterocytes. The deamidation reaction of TGc predominates at low pH which can occur in the duodenum and jejunum, which could explain why the ileum is often unaffected. [Non-enzymatic deamidation does not occur selectively, thus the gliadin polypeptides generated in the strongly acidic stomach do not serve as good epitopes (253)]. The TGc has been shown with immunogold electron microscopy to colocalise with toxic gliadin peptides on the apical surface of jejunal enterocytes, in the Golgi complexes, and on the basolateral cell membrane of enterocytes (Zimmer KP et al., oral communication). At the low pH of endosomes, the TGc might deamidate (and/or cross-link) gliadin.

Sollid et al. (256, 255) have attempted to explain how the recognition of foreign gluten peptides induces an enteropathy. Dietary gluten polypeptides (partially digested into smaller peptides) can reach the lamina propria even through healthy epithelial barrier; possibly in greater concentrations in infants with an immature barrier or during small intestinal infections. Indeed, gliadin may be directly toxic causing an increase in the permeability of epithelial barrier (125, Heyman et al., Fasano A., oral communication). Thus gluten peptides can reach substantial concentration in the mucosa and undergo deamidation. In genetically predisposed individuals, gluten peptides are presented by antigen presenting cells (B-cells, dendritic cells, macrophages and/or enterocytes) via the HLA-DQ2 or -DQ8 molecules. These cells efficiently activate both CD4+ T_{h1}-cells which drive inflammation and tissue remodeling and T_{h2}-cells which initiate antibody production against the gluten peptides. In the inflamed environment, TGc expression in lymphocytes and fibroblasts is increased enhancing extracellular deamidation as well as resulting in covalently cross-linked TGc-gluten peptide complexes (29, 261, 69, 207). Deamidation and cross-linking can also occur in the epithelial endosomes. If the ly-

sosomal degradation is incomplete, immunogenic, deamidated gliadin polypeptides cross-linked with certain TGc polypeptides might reach the basolateral surface of enterocytes. This complex can serve as neoantigen being recognised by both gluten-specific and TGc-specific B-cells.

Tolerance to soluble self-antigens, in contrast to tolerance to multivalent self-antigens, is regulated at the level of CD4+ T_h-cells. Both in healthy and affected individuals, B-cells specific for soluble self-antigens exist, but they fail to express autoantibodies because the CD4+ and áâ+ T_h-cells specific for these self-antigens are eliminated during the maturation of immune system. However, the deamidated gluten peptides-TGc complex can create a situation where a gluten peptide-specific T_h-cell provides help for a TGc-specific B-cell resulting in intense autoimmune response against TGc (Fig. 5). Namely, TGc-specific B-cells can selectively bind, internalise, and process TGc-gluten complexes via TGc-specific surface immunoglobulins. The gluten peptide-specific T-cells provide then cognate help for B-cell maturation, isotype switching, and autoantibody secretion.

According to this hypothesis, three types of Abs can be found in GSD: one directed specifically against gluten (gliadin), one against TGc, and one against parts of the cross-linked complex. Moreover, after the removal of gluten from the diet, the T-cell help discontinues, and the titre of circulating anti-TGc Abs declines. That gluten peptides trigger the AI response which is not maintained in the absence of this trigger even in the presence of large quantities of the autoantigen, makes a deep difference between GSD and other AI disorders.

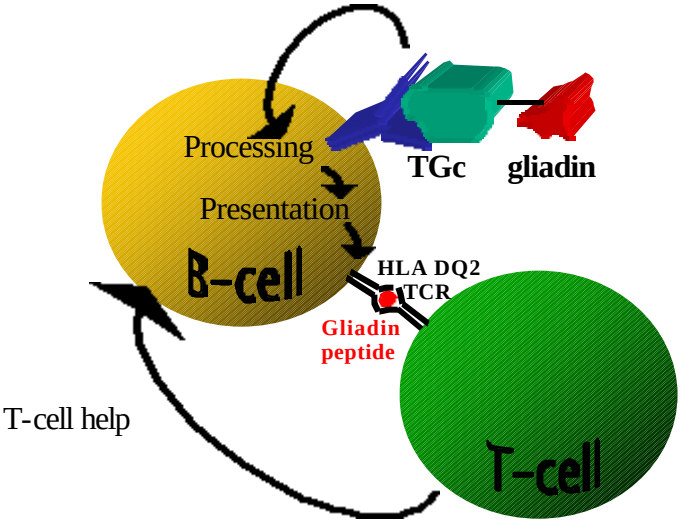

Fig. 5. Gliadin-specific T-cells can provide help to TGc-specific B-cells for Ab production. TGc-specific B-cells exist without producing Abs because TGc-specific T_h-cells were deleted during immune system maturation. However, a covalently cross-linked gliadin-TGc complex can be internalised by TGc-specific B-cells which then present the processed gliadin peptides with HLA DQ2 (or DQ8) resulting in efficient T_h-cell help and subsequently autoantibody secretion (256).

This theory would also explain why Abs to TGc are more disease specific than those to gliadin. The absorption of TGc-gliadin complexes can only occur in the small intestine. Thus the pathological events can only be driven in the small intestine, being aggravated by the inflammation of mucosa and increased permeability of epithelial barrier. In contrast, the immune reaction against gliadin can also result from the interaction of T- and B-cells in the regional lymph nodes, hence it does not require mucosal lesion. Self-proteins other than TGc such as calreticulin might also become cross-linked with gluten peptides resulting in autoantibody response (196, 238).

Whether the autoantibodies against TGc play a role in the pathogenesis of GSD or are just epiphenomena is not known. One suggestion is that the IgA autoantibodies

against TGc can inhibit its cross-linking activity (77, 115). A reduced deamidation ability might control GSD as unmodified gluten peptides are less efficient in maintaining enteropathy. Further, TGc activity is necessary for activation of TGF-â (218), and indirect inhibition of TGF-â activation by anti-TGc Abs might have profound effects on the differentiation of the intestinal epithelium (116). TGc is also involved in the cell adhesion and matrix stabilisation (279), thus the autoantibodies might also disturb the cell-cell and/or cell-extracellular matrix contact. These effects on epithelial differentiation could be responsible for the typical small intestinal histopathology and induce malabsorption. In addition, the anti-TGc Abs might be responsible for the extraintestinal manifestations of CD. Recently, also IgA autoantibodies directed against TGc have been described which activate the enzyme (Király R et al., personal communication). Thus the role of autoantibodies in GSD might be more complex than speculated above.

Small intestinal changes in both DH and CD are morphologically, functionally and clinically very similar, though DH patients show significantly less severe mucosal pathology and the clinical symptoms of enteropathy are usually absent or mild (96, see also 2.4.2 below). However, there is a profound difference in the Ab profiles: high affinity anti-TGe IgA maintained by gluten is present in DH patients but not in those suffering from CD (239). In addition, TGe is present in the upper dermal IgA aggregates typical of DH. A hypothesis for the etiology and pathogenesis of DH has recently been suggested by our research group (239).

Possibly TGc-gluten complexes initiate an IgA autoantibody response (256), but fail to produce high affinity anti-TGc immunoglobulins, so result initially in a silent CD. These Abs cross-react with TGe due to their high amino acid sequence similarity, but are of low avidity to it. After prolonged gliadin provocation (DH patients usually show symptoms later in life than CD patients), specific crossreacting Ab populations develop in patients who will go on to aquire DH. These Abs have a low affinity to TGc, but extremely high affinity to TGe (239). Whether they arise against TGe as a primary antigen or are the result of epitope spreading is unknown. Why only a proportion of patients develop specific Abs against TGe and why these patients show only a very mild form of enteropathy also remains to be elucidated.

The skin pathology may be evoked by the dermal deposition of circulating immune complexes containing IgA and TGe (239). Possibly the TGe is active, resulting in covalent cross-linking of the complex to certain dermal structural elements. This could be the basis for the stability of these immune complexes, as it is known that the IgA deposits in DH skin stay detectable up to a decade after the introduction of a completely gluten-free diet (96). It would also explain why it has not been possible to extract the IgA immune complexes from the skin of DH patients. Inflammation of the skin might eliminate the covalently bound immune complexes. Indeed, often the IgA granules are present perilesionally but not in areas of blister formation. This circulating immune complex hypothesis for DH is supported by a number of findings. Firstly that TGe is expressed in several tissues in the body including the kidney (Table I on page 19), thus the antigen might originate from organs other than the skin. Also the skin histology in DH has features in common with other dermatoses induced by circulating immune complexes (149), and although the main site of immune complex deposition is the upper dermis, they are also present in vessel walls (Fig. 9 on page 33). Further in DH, asymptomatic IgA immune complex depositions can be detected in the kidney (229), a situation often seen in systemic diseases caused by circulating immune complexes and indeed DH associated IgA nephropathy has been reported (120). Finally that Abs in DH

sera do not bind to the normal human papillary dermis again suggests that the deposits derive from circulating immune complexes. The factors that induce the classical distribution pattern of skin lesions in DH patients remain unknown.

2.4. Symptoms and signs

The clinical presentations of GSD are wide. Both CD and DH can be subdivided by different severity, as they can also present with unspecific or even absent clinical signs or symptoms. Further, epidemiological studies showed that the majority of GSD patients actually have very mild or atypical symptoms or often clinically silent disease (44, section 2.2.1). Patients with silent disease always have jejunal morphological evidence for CD. The term 'latent CD' is currently used for designation of patients fulfilling the following conditions: 1) no clinical symptoms of CD, 2) normal mucosa with traditional histology, 3) carrier status of HLA DQ2 or DQ8 molecules, 4) presence of EMA positivity and/or increased density of intraepithelial lymphocytes bearing ã/ä T-cell receptors and/or positive jejunal direct immunofluorescence for IgA (193, 285).

Table III. Symptoms, signs, and diagnosis of CD in alphabetic order (112, 56, 182).

GI symptoms and signs	ExtraGI symptoms and signs	Test results
• Abdominal distension, bloating, full abdominal contour (76%) • Bulky, loose, pale, stinking, or frothy stools (78%) • Chronic constipation (12%) • Chronic diarrhoea (68%) • Failure to thrive (81%) • General malaise (39%) • Glossitis • Loss of weight, anorexia (72%) • Nausea • Pancreatic insufficiency (if associated with CD, disappears after introduction of a GFD) • Recurrent abdominal pain (15-36%) • Secondary cow's milk intolerance (often associated with CD in early childhood, later usually disappears) • Short stature • Vomiting (54%)	• Amenorrhoea, infertility, late menarche, early menopause, habitual abortions • Ataxia • Bleeding (mostly skin bleeding) • Caliber reduction (sometimes loss) of hair • Cryptogenic hypertransaminasaemia • Cramps, tetany • Delayed onset of puberty • Dental enamel hypoplasia • Epilepsy with focal calcification in the brain • Fatigue, weakness • Finger clubbing • Hyposplenism • IgA nephropathy (mesangioproliferative glomerulonephritis) • Impaired male gonadal function • Iron deficiency anaemia, pallor • Lung fibrosis, alveolitis • Mood disturbances, changes • Myositis, (cardio-)myopathy • Oedema • Osteopenia, osteoporosis, or osteomalacia • Polyneuropathy • Recurrent aphthous ulceration	• quantity of stool fat • D-xylose absorption • 'Malabsorption pattern' with small intestinal x-ray • serum Ca^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+} • serum albumin, cholesterol, carotenes, vitamin A, ferritin • prothrombin time • Abnormal liver function tests • vitamin B_{12} and folic acid levels • urinary 5-hydroxy indoleacetic acid (5-HIAA) levels • Abnormal breath H_2 tests (sign for secondary lactase deficiency and/or bacterial overgrowth) • Abnormal intestinal histology • Positive serological tests (IgA or IgG EMA, AGA, and anti-TGc) • Typical HLA haplotype

The symptoms and signs of GSD are sometimes mixed with those of an associated disease. Such GSD-related conditions include type I diabetes mellitus and some other AI disorders (see Table XII on page 59 for details), selective IgA deficiency (54), Down's syndrome (41, 295, 236), and cystic fibrosis (183, 275).

2.4.1. Symptoms and signs of CD

CD commonly presents in infancy and early childhood with classical GI symptoms including chronic diarrhoea, vomiting, abdominal pain, distension and bloating, short stature, loss of weight, and failure to thrive (182). The disease may also present later in life with the symptoms of a typical malabsorption syndrom: loss of weight, abdominal distension and bloating, diarrhoea, steatorrhoea, together with abnormal tests of absorptive function (Table III, 56, 112).

CD is often monosymptomatic, thus one single manifestation like chronic diarrhoea, short stature or loss of weight of unknown origin may be the only presenting sign. In the majority of adult patients, the enteropathy presents with less typical symptoms like chronic iron deficiency anaemia, fatigue, constipation, habitual abortions, amenorrhoea, infertility, or ataxia. In these patients, the enteropathy is beleived to be localised only in the duodenum and the oral part of jejunum enabling the aboral part of jejunum and the ileum to adapt to the damage and loss of absorptive capacity of the oral parts of small intestine (144). As the damaged areas of small intestinal mucosa are distributed mosaically, the small bowel may perfectly compensate for the loss of resorption surface. Hence in certain individuals no symptom or sign indicating GSE with the exception of positive serology and small intestinal histology may occur (144). Moreover, pathological IgA deposits and increased number of intraepithelial T-cells bearing ã/ä receptor detectable only with immunohistochemical methods might be the only signs for intestinal affection (147, 243, 244).

The endoscopical picture of flat mucosa is suggestive of GSE, but it cannot replace the histological examination (Fig. 6 and 2.5.1).

2.4.2. Symptoms and signs of DH

The onset of skin symptoms is usually sudden but may also be gradual (76, 96). If untreated, the course of the disease is extremely chronic with spontaneous exacerbations and remissions (99). Long, asymptomatic periods are possible (96, 97, 205, 206). In the acute phase, the polymorphic skin symptoms resemble erythema multiforme or herpes

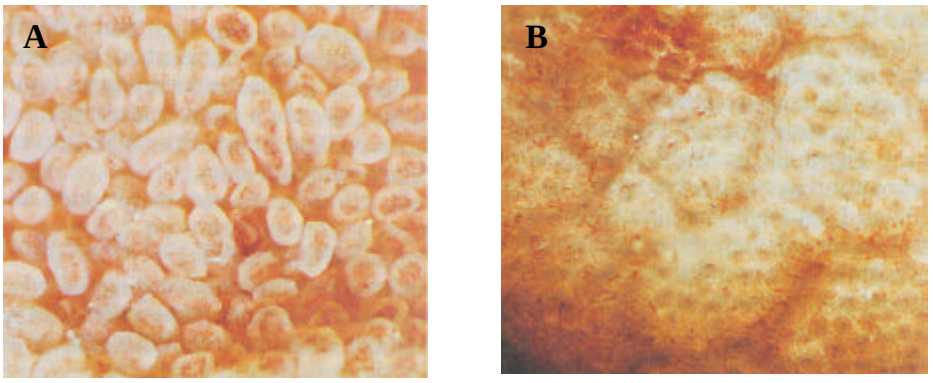

Fig. 6. Typical endoscopical picture of jejunal mucosa. **A**, normal jejunum. **B**, jejunum showing flat mucosa. (Reproduced from Ref. 40.)

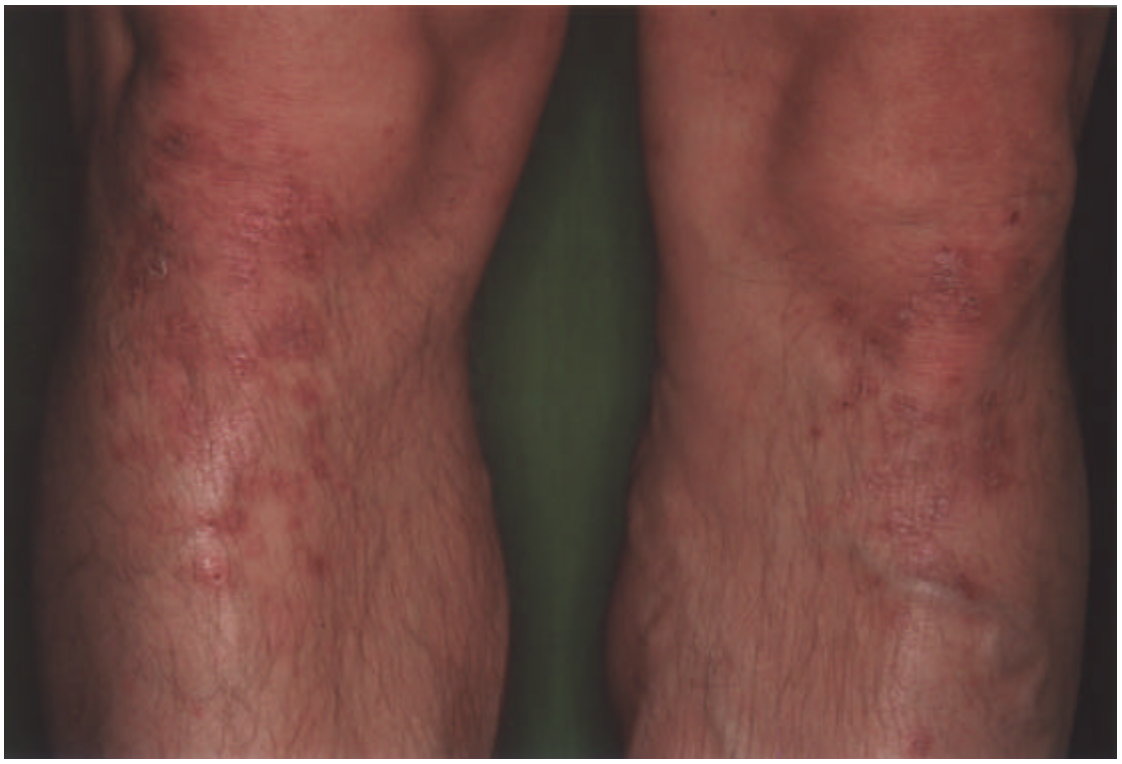

Fig. 7. Typical skin symptoms of DH: grouped, polymorphic, erythematous, excoriated papules distributed symmetrically over the knees. Blisters are not necessarily present, the distribution of the rash is usually more suggestive of the diagnosis than the finding of vesicles.

virus infections: grouped or clustered, erythematous, urticarial, exsudative, excoriated papules and plaques appear typically associated with small vesicles, but bullous lesions develop rarely (Fig. 7). In the chronic phase, excoriated papules based on postinflammatory, hypo- and/or hyperpigmented macules are common, it may be impossible to find an intact vesicle because of the excoriation. Occasionally, annular and gyrate patterns of lesions with characteristic central clearing and vesicles at the erythematous margins of large plaques may be seen. The eruptions most often affect the extensor surfaces of the major joints (elbows, knees), the scalp (occiput, facial hairline), shoulders and posterior axillary folds, sacrum, buttocks, and natal cleft. However, any other sites of the body may be involved including the oral mucosa. The commonest sites are the elbows (95%), the next commonest the buttocks, the third most frequent the knees. Interestingly, the disease rarly appears on the head, but it is often affected later on during exacerbations. The lesions tend to occur at sites of pressure from clothing, e.g. under tight belts. Pruritus is usually predominant, extremely severe, may even be disabling, but the general health is never affected. In addition, burning or stinging sensations may also accompany the condition (151). Due to the intensive scratching, secondary impetiginisation is common.

Beside the above alerting lesions, there are also discrete palmoplantar symptoms which are usually not noted by the patient: reddish-brown or dark lilac, mottled, irregular shaped spots of 1-5 mm diameter together with pinpoint-like macules resembling purpuras; referred to as pseudopurpuras (210, 151, Fig. 8). The reddish-brown spots are remnants of small, flat, haemorrhagic vesicles. These are only present for a few weeks, do not accompany every relapse or correlate with the severity, but are important because

they can confirm the diagnosis or lead the physician's attention to the possibility of DH (210, 151). They are found mainly in regions exposed to various mechanical irritations and pressures, i.e. on the flexor surface of fingers, fingertips, and palms, rarely also on the plantar surface of toes, but never on the dorsal or extensor aspects (Fig. 8). The lesions affect the right hand, particularly the right index finger, more intensively than the left side (151).

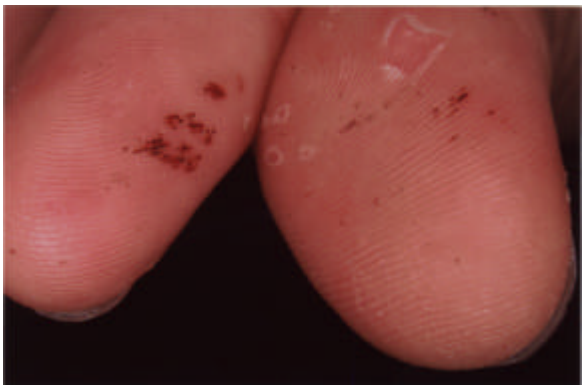

Fig. 8. Reddish-brown, irregular pseudopurpuras of 1-5 mm diameter together with scaling on the flexor surface of thumbs of a patient with DH. Note that the lesions are more extended on the right thumb.

Although CD and DH are closely related diseases, less than 10% of patients with DH have gastroenterological symptoms suggestive of GSE (96, 232). The enteropathy in DH is morphologically, functionally and clinically very similar to that in CD (96). GI symptoms are usually absent or so mild that the DH patients are unaware of them, but inflammatory small bowel changes can often be found by histological examination (95, 96, 167, 230). In a number of DH patients, no intestinal lesions can be found by traditional histology; however, pathological IgA deposits and increased number of ã/ä receptor positive intraepithelial T-cells are almost always present (2.5.2, 147, 244). In certain rare cases, the whole GI and/or extragastrointestinal symptom spectrum of CD may be detected, but the skin lesions always predominate.

2.5. Diagnosis

2.5.1. Endoscopy

The endoscopic markers for CD are 1) the mosaic appearance of the duodenum, 2) the reduction or loss of duodenal (Kerkring's) folds, 3) scalloped duodenal folds in the descending duodenum, 4) nodular mucosal pattern, and 5) visible underlying submucosal vessels (200, Fig. 6). In contrast to their high specificity (>90%), the sensitivity of these markers is relatively low (15-76%) if only one of them can be observed, but the more markers are present, the higher is their sensitivity and thus positive predictive value (200). Nevertheless, histological diagnosis should always confirm the clinical suspicion.

2.5.2. Histological examinations

The diagnosis of GSE was based in the 1970-80s on the characteristic histological changes (villous atrophy, intraepithelial lymphocytosis, crypt hyperplasia) seen in small intestinal biopsies, followed by the regeneration of the mucosa after a GFD, and relapse during subsequent gluten challenge (203). However, during the last two decades it became clear that when the original biopsy shows severe enteropathy, and the clinical symptoms disappear after introduction of a GFD, the subsequent gluten challenge produces positive results in 95-99% of the patients. Accordingly, the criteria have been

revised, and gluten challenge is no longer considered mandatory if there is a clear-cut clinical response with relief of all symptoms within weeks (283). All children diagnosed in the first 2 years of life, those individuals in whom the diagnosis was doubtful or were asymptomatic and where there may have been other explanations for the histological abnormalities, a gluten challenge is still useful to confirm the diagnosis which has serious, life-long consequences.

Today it is evident that not only severe villus atrophy but also other histological changes are consistent with the diagnosis of GSE. Being aware of this, Marsh has subdivided the (unspecific) traditional histological changes seen in GSE (198) into five lesion groups (Table IV). Many disorders (also others than listed in Table IV) can cause flat and/or infiltrative mucosal lesions. This is why the demonstration of mucosal regeneration after introduction of a GFD is essential for differential diagnosis, and sometimes a gluten challenge is also needed. In addition, the symptoms of GSE can be induced by several factors including coexisting nutritional deficiencies (e.g iron deficiency), GI diseases (e.g. pancreatic insufficiency), metabolic stress (e.g. surgery), malignancies (e.g. intestinal lymphomas), and GI infections [e.g. adenovirus 12 infection (141, 13)].

Table IV. The main five lesions associated with GSE together with some disorders of differential diagnostic importance (198). The type 0 lesion designates normal mucosa (latent GSE). Type 1 lesion means that the ratio of (CD3+ and ã/ä+) mucosal intraepithelial lymphocytes to enterocytes is elevated. Type 2 lesions show, in addition to an infiltration with intraepithelial lymphocytes, hyperplastic changes of villi. Type 3 designates the typical (but still unspecific) 'flat mucosa', and type 4 labels the 'villus atrophy' being specific for GSE, but frequently associated with refractory sprue or intestinal lymphoma.

Lesion	Preinfiltrative (type 0)	Infiltrative (type 1)	Infiltrative hyperplastic (type 2)	Flat destructive (type 3)	Atrophic hypoplastic (type 4)
GSE	+	+	+	+	+
Infective enteropathy	+	+	+	+	
Tropical sprue	+	+	+	+	
Graft-versus host disease		+	+	+	
Transient food sensitivities		+	+	+	

The symptoms and their severity are not related to the degree of mucosal pathology, but depend on the extent of small intestinal lesions which are often patchy (198, 244, 147). Hence it is useful to take biopsy from asymptomatic individuals being positive with serological tests, and the finding of 'normal mucosa' by traditional histology does not exclude the possibility of GSE. Immunohistochemical methods can reveal latent GSE within 'normal mucosa': pathological IgA deposits, high number of immunoglobulin-containing cells, and increased density of ã/ä receptor positive intraepithelial T-cells detectable only with these methods might be the only signs of alteration in CD or DH (198, 147, 243, 244, 260). The elevated absolute number of ã/ä receptor bearing intra-

epithelial T-lymphocytes is relatively constant, does not depend on the gluten consumption (243), and is a specific sign for GSE.

The diagnosis of DH is based on the immunohistochemical detection of the typical IgA deposits in the papillary dermis found upon perilesional biopsy (96, 276, 239, Fig. 9). Traditional skin histology can only suggest but not prove the disease: microabscesses occur in the dermal papillae containing mainly neutrophils but often also eosinophils. In addition, leukocytoclastic vasculitis with lymphohistiocytic cell infiltrates may be present in the upper dermis together with immunoglobulin and complement depositions (96, 225). IgA deposits can also be found in the uninvolved skin during spontaneous or dapsone-induced remission (96), and only disappear in one fifth of patients after a decade (on average 13 years) of a strict GFD (99). A small intestinal biopsy is useful but not crucial for the diagnosis of DH, because the skin and GI symptoms do not correlate, and intestinal lesions can be distributed in a segmental pattern, thus the detection of normal mucosa by traditional histology does not exclude the possibility of DH (96, 167).

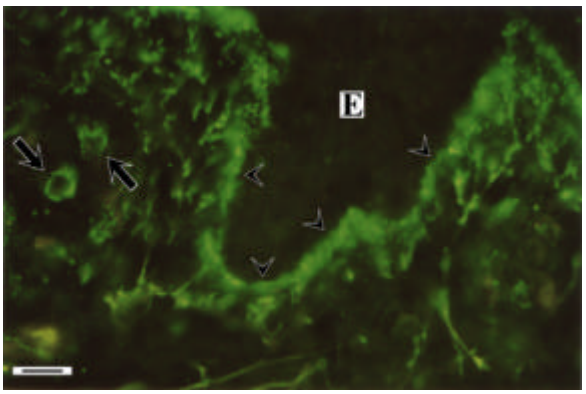

Fig. 9. Direct immunofluorescence on a skin specimen from a patient with DH at high magnification (× 200; bar=25 ì m). Bound IgA was detected by á-chain-specific, fluorescein isothiocyanate-conjugated rabbit anti-human IgA Abs. The IgA immune complexes resemble the falling snow in the papillary dermis. The IgA deposits are most intensive at and just below the basement membrane (arrow heads). IgA can also be detected in the blood vessels (arrows). **E**, epidermis.

2.5.3. Serological tests

Although the intestinal histology is still essential, the less invasive and cheaper serological tests may be helpful in the diagnosis of GSD. In addition, they are more suitable for follow-up of patients and for screening of asymptomatic individuals.

The two major types of Abs produced by the immune system in GSD are directed against gliadin and TGc. However, also other Abs have been reported including those against TGe (239), calreticulin (168), desmin (264), ovalbumin (196), â-lactoglobulin (196), actin (52), and other yet unidentified nuclear, cytosolic, and extracellular proteins (194, 196). In the early 1980s, the anti-gliadin ELISAs became widespread for diagnosis of GSE, and an association between GSD and circulating IgA EMA was demonstrated (48). Since 1997, anti-TGc Abs have also been assayed. The currently used serological tests are isotype-specific, and detect mainly the disease-specific IgA Abs directed either against gliadin (anti-gliadin antibody test = AGA test) or tissue antigens. The tissue antigens currently used for diagnostic purposes include the monkey endomysial antigen (EMA test), the reticulin (ARA test performed on monkey liver, kidney, and stomach), and cytoskeletal proteins from the human umbilical cord (umbilical cord test).

IgG Ab based tests are generally either not sensitive or specific enough for GSD diagnosis in patients with normal serum IgA levels (54). However, IgG based tests are

useful for the diagnosis in patients with selective IgA deficiency, which is known to be associated with GSD (54, 42).

Table V. Sensitivity and specificity of serological tests used for diagnosis of GSE in untreated CD patients with normal serum IgA levels. Serological tests based on TGc are not included.

Test	IgA class		IgG class	
	Sensitivity	Specificity	Sensitivity	Specificity
AGA (ELISA)	30-100% (~78%, children: ~95%)	30-100% (~94%)	46-100% (~90%, children: ~95%)	67-100 (~80%, children: ~85%)
EMA (monkey)	80-100 (~95%, children: ~100%)	95-100 (~100%)	~66%	~50%
ARA (rat)	29-100 (~72)%	95-100 (~99%)	~66%	~50%
Umbil. cord tissue or cells (human)	70-100 (~90%, children: ~95%)	95-100 (~100%)	data not available	data not available

The IgA and IgG AGA tests are currently based on a commercially available ELISA technique. Their sensitivity and specificity vary from 30% to 100% (Table V, 195, 196, 212). The titre of AGAs is age dependent, thus the AGA ELISA has different cutoff values for children below and above two years of age. While in children, the result of AGA correlates satisfactorily with the mucosal pathology; in adults, AGA can be found in healthy individuals or absent in untreated GSE. With current techniques and standardisation efforts, a sensitivity and a specificity of >90% (in children under two years of age even >95%) can be achieved with IgG and IgA AGA tests (43), but both IgG and IgA AGA are should be tested at the same time.

The IgA-class endomysial Ab (EMA) test (Fig. 10) is considered to be the serological method of choice with a higher sensitivity and specificity when compared to the IgA-class ARA (on rat tissues) or AGA tests (119, 170, 146). ARA on other monkey tissues than the oesophagus gives similar specificity and sensitivity, but the oesophagus is easier to interprete and more suitable for differential diagnosis of bullous AI skin diseases (119, 170). EMA is a less sensitive indicator in children under the age of 2 years (182). EMA is found in approximately 70% of untreated patients with DH (22, 47) and in almost all untreated patients with CD (47). IgG-class EMA test can be performed in patients with IgA deficiency, but results are more difficult to interprete because of the high IgG background usually present in the oesophagus.

As the EMA test is usually performed on expensive oesophagus sections from endangered primates, is laborious and time consuming, and is subjective in borderline cases, a demand for alternative test methods exists. The use of rabbit, rat or guinea pig

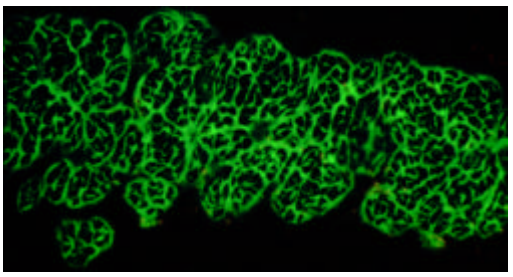

Fig. 10. Positive EMA test. IgA antibodies from a CD patient show typical honeycomb-like, endomysial staining with indirect immunofluorescence on a monkey oesophagus section (×80 magnification).

oesophagus, jejunum or other tissues results in considerable loss of sensitivity (119). However, human umbilical cord sections (171) or immortalised human umbilical vein endothelial cells (287) are alternative substrate tissues offering similar results.

Ultrastructurally, the EMAs (and the jejunal Abs) from CD and DH bind to a non-fibrillar, amorphous component next to collagenous-reticulin fibrils of the connective tissue connecting adjacent smooth muscle cells, smooth muscle bands, and perimysial elastic tissue (148, 150). TGc has now been shown to be the common target of EMA, ARA, and the jejunal Abs in both CD and DH supporting ultrastructural observations (162, 163). Using double-staining investigations, anti-TGc MAb binding patterns were found to be identical with those seen with CD patients' sera, and CD patients' sera blocked the binding of anti-TGc MAbs (163). Extraction of TGc from the tissues abolished the staining patterns, but they were elicited again after readdition of TGc (163). No GSD patient sera reacting with wild-type mouse tissues elicited any endomysial, reticulin or jejunal bindings in TGc-/- mice, but they showed typical endomysial, reticulin or jejunal patterns with the TGc-/- mouse tissues after coating with human TGc (162 and Korponay-Szabó IR et al., personal communication). An ELISA test for GSE based upon the commercially available guinea pig TGc (gpTGc) gave, after optimisation using calcium-activation (71, 259) high sensitivity and specificity (above 90%). However, as the amino acid sequence identity between guinea pig and human TGcs is 82.8% (133, 104), there may be patient Abs directed against epitopes of human TGc (hTGc) not conserved in the gpTGc.

2.5.4. Other tests

In addition to the histology, the presence of malabsorption can also be examined with cheap, simple, and noninvasive methods. The D-xylose test is highly sensitive for CD, and although it is not specific, it correlates excellently with the histological grade of GSE (26, 125). It is also useful in the follow-up. The H_2 breath test is not sensitive and specific enough for diagnosis of GSE, but it is important because the results show very good correlation with the severity of the mucosal damage and extent of affected intestine (25). Thus it is useful for follow-up or decision on supportive therapy. The leukocyte migration test is a suitable tool for detection of dietary failures (125, 126), but being laborious, it is not widely used.

DH symptoms can be induced by a iodine-rich diet or a skin test using iodine if necessary.

Laboratory tests listed in Table III, osteodensitometry etc. should be performed partly for additional information to the diagnosis (concomittant disorders may have therapeutic consequences), partly for exclusion of other GI diseases. Determination of the HLA haplotype can also be helpful.

The therapy, complications, and prognosis of CD and DH should be described in a work about GSD. However, being not related to the experimental part, these topics were omitted from this thesis.

3. AIMS

In 1997, Dieterich et al. suggested TGc was the main endomysial autoantigen of CD (69, 72). After optimisation using calcium-activation, an ELISA test based upon the commercially available gpTGc gave high sensitivity and specificity for CD (above 90%) (71, 259). Thus the aim of these studies was to confirm the significance of this finding for diagnostic (screening) and follow-up purposes, and further studies were planned to optimise the assay and especially to detect potential disadvantages. The project was divided into three parts.

1. The guinea pig TGc (gpTGc) was used to investigate the nature of autoantibodies directed against TGc (effect of a GFD, association with the EMA signal). This project will be designated as 'gpTGc ELISA study'. The sensitivity and specificity of the gpTGc ELISA was determined and compared to the EMA test for diagnosis of GSD. In addition, follow-up sera were studied to detect titre changes during a GFD, and inhibition of endomysial staining were performed with EMA-positive sera with low Ab titres against TGc to see whether autoantigens other than TGc may be responsible for EMA positivity.
2. It was tested whether the performance of the TGc ELISA could be improved by using human TGc (hTGc) as antigen, as patient sera not recognised by the gpTGc ELISA may have Abs directed against epitopes of hTGc not conserved in the guinea pig enzyme ('hTGc ELISA study'). For this study, hTGc was expressed recombinantly, and an ELISA based on the purified protein was established for IgA anti-hTGc Abs. The results of this assay were compared to those from the gpTGc ELISA and the EMA test on monkey oesophagus.
3. Finally, the specificity of the TGc ELISAs was further determined using serum samples from patients having a wide spectrum of different diseases, because in initial studies, certain AI patients without GSD were observed to give positive results in the TGc ELISAs suggesting a general role for TGc in AI processes ('AI study'). Also patients with non-AI diseases involving enhanced apoptosis and cell lysis and/or putative secondary AI processes e.g. hepatitis or malignancies, where release of TGc could theoretically lead to anti-TGc Ab formation, were included.

The studies have been in part published (240, 241), and I received permission for republication of figures, tables and parts of text in this thesis from the copyright owners (Karl Demeter Verlag and American Association for Clinical Chemistry).

The major part of the study was performed in the Institute for Biochemistry II., Medical Faculty, University of Cologne.

4. METHODS

4.1. The gpTGc study

4.1.1. Sera and patients

The patients had been examined at the Gastroenterological Departments of Clinic of Internal Medicine II or Clinic of Pediatrics II and the Department of Dermato-Venereology of the Semmelweis University. The CD diagnosis was confirmed by jejunal biopsy, whereas DH was confirmed by skin biopsy. Sera were obtained from 47 patients with DH, 120 with CD, 96 with non-CD gastrointestinal (GI) diseases, and 117 with other diagnoses (Table VI). Thus altogether 380 serum samples were used. The DH sera consisted of 30 samples from patients with untreated DH, 16 from patients on a complete or incomplete gluten-free diet (GFD), and 1 from a patient on a gluten challenge (GCH). The CD sera consisted of 37 samples from patients with untreated CD, 72 from patients on a complete or incomplete GFD, and 11 on a GCH (Table VI). The CD and DH sera also included 2-3 follow-up samples from 13 patients which were taken before and in different time points (5-49 months) after the introduction of a GFD. All untreated GSD patients were positive for EMA with the exception of two untreated DH patients (Table VI). To compare the sensitivity of the EMA test with that of the gpTGc ELISA, we tested several sera from patients on a complete or incomplete GFD or during a GCH which may contain significantly reduced amounts of Abs against TGc. In this study, GCH was given only to patients with uncertain, ambiguous or contradictory jejunal histology and/or EMA tests before reexamination. Mean ages and sex ratios of the patients are detailed in Table VII. Control patients with non-CD GI diseases had the following diagnoses (number of patients in parentheses): chronic pancreatitis (1), diarrhea after administration of broad spectrum antibiotics (1), undiagnosed, chronic diarrhea (4), psychosomatic diarrhea (1), toddler's diarrhea (8), infectious diarrhea (3), diarrhea due to immunodeficiency (1), irritable bowel syndrome (2), post-enteritis diarrhea (4), post-enteritis lactose intolerance (2), primary adult lactose intolerance (11), fructose intolerance (2), isomaltase deficiency (1), multiple food protein intolerance (3), soy protein intolerance (1), cow's milk protein intolerance (22), cow's milk and egg protein intolerance (2), gastroesophageal reflux (5), giardiasis (6), helminthiasis (3), habitual constipation (1), retarded growth (<3 percentile) due to impaired intestinal absorption of various origin (12). Other diagnoses were: anorexia nervosa (1), atopic dermatitis (1), bullous phototoxic contact dermatitis (1), retarded growth due to chronic adenoiditis (1), retarded growth due to feeding failure (2), retarded growth of other than GI origin (19), familiar retarded growth (3), somatomental retardation (1), cystic fibrosis (1), Silver-Russel syndrome (1), systemic lupus erythematosus (1), insulin dependent diabetes mellitus (1), primary liver carcinoma (1), prolonged microcyter anemia (5), cholelithiasis (1), neuroblastoma (1), herpes gestationis (1), ichthyosis (31), IgA-pemphigus (3), linear IgA bullous dermatosis (3), pemphigus foliaceus (1), pemphigus seborrhoicus (1), urticaria (1), cryofibrinogenemic skin vasculitis (1), CD patients' healthy relatives (21), healthy individuals (13). No patient or healthy individual in this study had IgA-deficiency. All serum samples were stored at -78°C until assayed.

Table VI. Number and percentage of total, EMA positive (EMA⊕) and TGc ELISA positive (TGc⊕) serum samples and serum Ab titres against TGc

Diagnosis	TN of SS	% of SS	EMA⊕ SS		TGc⊕ SS		TIT (AU)
			No.	% of TN	No.	% of TN	
CD	120	31.6	73	60.8	65	54.2	18.7
CD, on a GFD	72	19	34	47.2	27	37.5	10.3
CD, untreated	37	9.7	37	100	35	94.5	70.6
CD, on a GCH	11	2.9	2	18.2	3	27.3	8.2
DH	47	12.4	36	76.6	35	74.5	26.3
DH, on a GFD	16	4.2	7	43.8	7	43.8	13.2
DH, untreated	30	7.9	28	93.3	27	90	37.3
DH, on a GCH	1	0.3	1	100	1	100	84.8
Non-CD GI diseases	96	25.2	0	0	0	0	8.2
Others	117	30.8	0	0	3	2.6	8.5
All samples	**380**	**100**	**109**	**28.7**	**103**	**27.1**	**9.3**

Abbreviations: SS: serum samples; EMA⊕: EMA positive; TGc⊕: TGc ELISA positive; No.: number; TN: total number; TIT, median serum Ab titres against TGc in AU.

Table VII. Age at the time of blood sampling (years) and sex of patients

Diagnosis	Male/female	Mean age	Minimum age	Maximum age
CD	50/70	11.6	0.8	66
CD, on a GFD	31/41	9.4	0.8	26
CD, untreated	13/24	16.7	1.3	66
CD, on a GCH	6/5	9.6	4.2	16
DH	24/23	30.2	8.8	71
DH, on a GFD	8/8	25.7	10.3	45
DH, untreated	15/15	32.9	8.8	71
DH, on a GCH	1/0	19	19	19
Non-CD GI diseases	50/46	5.7	0.4	31
Others	52/65	16.2	0.7	78
All samples	**176/204**	**13.8**	**0.4**	**78**

Abbreviations: GCH, gluten challenge; GFD, gluten-free diet; GI, gastrointestinal.

4.1.2. Indirect immunofluorescence (EMA test)

Serum IgA Abs were measured by an indirect immunofluorescence method (54). All serum samples were diluted 1:5 (or up to 1:40 where necessary) in phosphate-buffered saline (PBS, pH 7.4). Cryostat tissue slides (6 µm) of the aboral part of the monkey

(*Cercopithecidae* family) oesophagus were used as antigen. Bound IgA was detected by á-chain-specific, fluorescein isothiocyanate-conjugated rabbit anti-human IgA Abs (1:40 in PBS; Dako). All sera used in these studies were indisputably negative or positive for IgA EMA.

4.1.3. ELISA

The ELISA method will be described under 4.2.7.

4.1.4. Inhibition of EMA

Nine sera from CD or DH patients with EMA positivity and low serum IgA Ab titres against the gpTGc were diluted to various concentrations (between 1:5-1:40, to the highest still positive concentration) in PBS, pH 7.4 and 50 mM Tris/HCl, pH 7.5, and incubated 2 hours and/or overnight in a shaking incubator with 20 ìg gpTGc (SIGMA) dissolved in 50 mM Tris/HCl, pH 7.5 at room temperature. The treated serum dilutions were then examined for EMA on monkey oesophagus sections as described above.

4.1.5. Statistics

The statistical methods will be described below (4.2.8).

4.2. The hTGc study

4.2.1. Sera and patients

The patients overlapped in part with those described above (4.1.1). Serum samples were taken from 71 patients with untreated GSD (33 with DH and 38 with CD), 26 with non-CD GI diseases (such as Crohn disease, food hypersensitivity, food intolerance, intestinal infection, reflux oesophagitis, non-CD diarrhoea, and alimentary dystrophy), and 27 with other diagnoses, such as AI diseases (systemic lupus erythematosus and diabetes mellitus type I), different skin disorders (pemphigus foliaceus, ichthyosis, and urticaria), cholelithiasis, hepatosplenomegaly, retarded growth of other than GI origin, as well as healthy controls. The mean ages and sexes of patient groups are presented in Table VIII. To obtain data on the sensitivity of the TGc ELISA, we included in the current study sera from 16 treated patients (patients on a GFD). All serum samples were stored at -78 °C until assayed.

Table VIII. Age at the time of blood sampling (years) and sex of patients

Diagnosis	Male/female	Mean age	Minimum age	Maximum age
CD	20/18	18.5	3	51
DH	13/20	30.2	6	74
Non-CD GI diseases	14/12	17.4	1	78
Others	14/13	17.0	12	53
All samples	**61/63**	**20.8**	**1**	**78**

4.2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-PAGE was performed according to the method of Laemmli (172) using a 12% polyacrylamide separating gel with a 5% polyacrylamide stacking gel. Samples were reduced by addition of 2% (v/v) 2-mercaptoethanol. Proteins were detected either by staining with Coomassie Brilliant Blue R (Serva) or by immunoblotting after electrophoretic transfer to a nitro-cellulose membrane (Protran®, Schleicher & Schuell) (269). After protein transfer, the membranes were stained with Ponceau S (Serva), then blocked with 50 mM Tris, 150 mM NaCl, pH 7.4 (TBS) containing 5% non-fat milk powder for 75 min. at room temperature. The blocked membrane was incubated with a mixture of two mouse monoclonal Abs (MAbs) against TGc (specific for TGc, but cross-reacting both with human and gpTGc, Neomarkers, designated as clones CUB7402 and TG100) diluted 1:2000 in TBS containing 5% non-fat milk powder and 0.05% Tween 20 (Sigma) for 1.5 hours at room temperature. For detection of bound mouse Abs, membranes were incubated with horse radish peroxidase labelled rabbit Abs directed against mouse immunoglobulins (Dako), diluted 1:2000 in TBS/Tween containing 5% non-fat milk powder for 1 hour at room temperature. Bound secondary Abs were detected using the enhanced chemiluminescence system (ECL Kit, Amersham). GpTGc (Sigma) was used each time as a positive control.

4.2.3. Recombinant expression of hTGc

4.2.3.1. Construction of the vector

The episomal eukaryotic expression vector pCEP-Pu/BM40SP, produced from pCEP4 (Invitrogen) (158), was modified to introduce a sequence encoding the Strep II tag (Institut für Bioanalytik, Germany) and a stop codon into the multiple cloning site. The primers 5'-GGCCGCATGGAGCCATCCA CAATTCGAAAAGTA and 5'-GGCCTACTTTTCGAATGCTG GATGGCTCCATGC were annealed together and introduced into the Not I site thus constructing a vector (pCEP-Pu/BM40SP/C-Strep) producing a carboxyterminal Strep II fusion protein suitable for streptavidin affinity purification by a StrepTactin® (Institut für Bioanalytik, Germany) affinity column as described before (246). We received the full-length human TGc cDNA (accession number M55153, cloned in pSP73) from Dr. Daniel Aeschlimann, Division of Orthopaedic Sur-

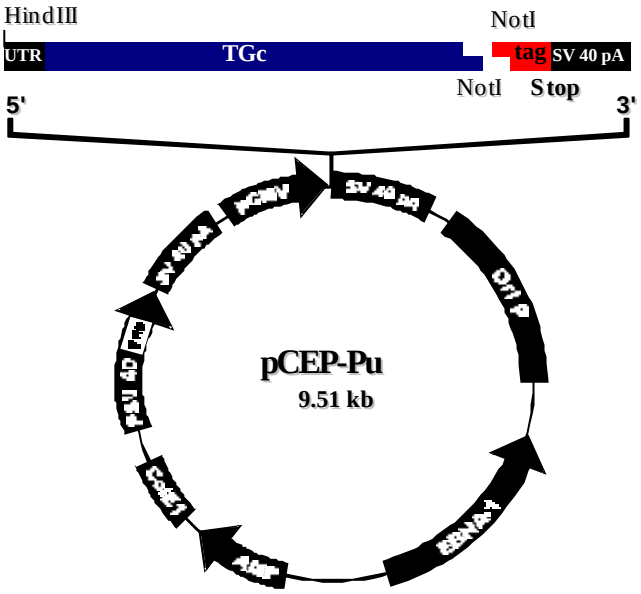

Fig. 11. The pCEP-Pu/TGc/C-Strep episomal eukaryotic expression vector which enabled to express recombinant human TGc in human embryonic kidney cells where its production was under the control of the intermediate early CMV promoter. The TGc protein was fused in frame to a C-terminal Strep II tag so that it could be readily purified from cell lysates by affinity chromatography.

gery, University of Wisconsin. This cDNA was amplified by polymerase chain reaction (PCR) using the 5'-primer 5'-ATTAAGCTGCCCGCCACCATGGCCGAGAGCT GGTC, and the 3'-primer 5'-TAAGCGGCCGCGGGGCCAATGATGACATTC. The 5'-primer introduced a new Hind III restriction site and a Kozak's translation initiation sequence, the 3'-primer inserted a new Not I restriction site and removed the stop codon. The Hind III/Not I restriction enzyme digested PCR product was purified and inserted at the same restriction sites of the pCEP-Pu/BM40SP/C-Strep, in order to obtain the final expression vector pCEP-Pu/TGc/C-Strep. The correct insertion and sequence of the full construct was verified by cycle sequencing using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit and the products were resolved on an ABI Prism 377 Automated Sequencer (Perkin-Elmer/Applied Biosystems).

4.2.3.2.Transfection, cell culture and protein purification

Human embryonic kidney cells (293-EBNA; Invitrogen) were transfected episomally with pCEP-Pu/TGc/C-Strep. The cells were cultured in Dulbecco's MEM NUT MIX F-12 (Life Technologies) medium containing 10% foetal bovine serum (Life Technologies), 1% L-glutamine (Life Technologies), 200 IU/ml penicillin (Life Technologies), and 200 mg/l streptomycin (Life Technologies). Cells were selected with 0.5 mg/l puromycin (Sigma).

After the removal of the media, the cells were washed with cold (4 °C) 0.25 M sucrose and lysed mechanically in the same solution. The lysate was cleared of particulate material by centrifugation at 27,200*g* for 30 min at 4 °C, followed by ultracentrifugation of the supernatant at 210,000*g* for 60 min at 4 °C. The supernatant was filtered with cheesecloth and 1 mM phenylmethylsulfonyl fluoride (PMSF, Fluka) was added as proteinase inhibitor; 12 ml of the supernatant was then passed over a StrepTactin affinity coloumn of 3 cm^3 volume equilibrated with sterile filtered 50 mM Tris-HCl, pH 7.5, containing 1 mM ethylenediamine tetraacetate (EDTA) at 4°C and at a flow rate of 0.4 ml/cm^2/min. After extensive washing with equilibration buffer containing 1 mM PMSF at a flow rate of 0.9 ml/cm^2/min, the protein was eluted with equilibration buffer containing 1 mM PMSF and 2.5 mM desthiobiotin (Sigma) at a flow rate of 0.4 ml/cm^2/min. Fractions (2 ml) were collected. The purification was controlled by Coomassie-stained SDS-PAGE and immunoblotting with MAbs against TGc as described above. The protein concentration was estimated by SDS-PAGE and measured using the bicinchoninic acid protein assay reagent (Pierce) according to the supplier's protocol.

4.2.4. TGc activity assay

TGc activity was measured by incorporation of [1,4-^{3}H]putrescine (Amersham) for 30 min at 37 °C as described previously (4), the buffer used contained 22.5 mM dithiothreitol to reduce any oxidised sulfhydryl groups important for catalytic activity.

4.2.5. Mass spectrometry

Mass spectrometry was performed by matrix-assisted laser desorption using a Bruker Reflex III instrument equipped with a high mass detector for linear detection. Sinapinic acid was used as the matrix, and external calibration was carried out using singly, doubly, and triply charged molecular ions of protein A.

4.2.6. EMA test

The EMA test was performed as described above (4.1.2).

4.2.7. ELISA

The ELISA method was similar to the calcium-activated test described previously (71, 259). Ninety-six-well microtitre plates (Nunc MaxiSorp) were coated with 1 µg gpTGc (Sigma) or hTGc in 100 µl of 50 mM Tris-HCl, pH 7.5, containing 5 mM $CaCl_2$ per well at 4 °C overnight (at least 9 h). No blocking was used. After each step, the wells were washed with 50 mM Tris-HCl containing 10 mM Na_2-EDTA and 0.1% Tween 20 (TET). Sera were diluted to various concentrations with TET, and incubated on the plates for 1.5 h at room temperature. Bound IgA was detected by peroxidase-conjugated Ab against human IgA (Dako), diluted 1:4000 in TET and incubated for 1 h at room temperature. The colour was developed by the addition of 100 µl of 60 µg/ml 3,3',5,5'-tetramethylbenzidine substrate in 100 mM sodium acetate, pH 6.0, containing 0.015% H_2O_2 for 5 min at room temperature. The reaction was stopped by adding 100 µl of 20% H_2SO_4. The absorbance was read in an ELISA reader at 450 nm.

The amount of protein and the serum concentrations used in the test were optimised. All serum samples were examined in triplicates, and triplicates of a negative and a positive reference serum as well as a buffer blank were included in each assay. The Ab concentrations were expressed in arbitrary units (AU), i.e., as percentages of the positive reference serum.

To obtain data on the effects of calcium activation, an experiment in which wells were coated with human TGc without $CaCl_2$ in the coating buffer was also performed.

4.2.8. Statistics

Absorbances (and thus titres given in AU values) did not show gaussian distribution; thus for statistical description of titres from the different patient groups, medians with their 95% confidence intervals (95% CIs) (98) are presented, and for comparison, the Mann-Whitney nonparametric, unpaired, two-tailed test was used (286). For describing correlation of titres, the Spearman correlation coefficient with its 95% CI and correlation analysis for unpaired data of nongaussian distribution were used calculating two-tailed *P* values (98, 286). For comparison of titres in the calcium-activated and unactivated hTGc ELISA, the Wilcoxon two-tailed signed-rank test was performed (286). For description and comparison of the two ELISA systems, the receiver operating characteristic (ROC) curves and the areas under the ROC curves (AUCs) with their 95% CIs are presented (66, 209, 121). For calculating confidence intervals of AUCs, a bootstrap technique, the bias-corrected and accelerated (BC_a) confidence interval method (209, 121), was applied in addition to the most frequently used method (66) because it is more appropriate for describing confidence intervals of AUCs that are very close to the maximum (1.0).

4.3. The AI study

4.3.1. Sera and patients

The sera were obtained from the Gastroenterological Departments of Clinic of Internal Medicine II or Clinic of Pediatrics II, the Department of Dermato-Venereology of

the Semmelweis University, the Departments of Internal Medicine I-IV of the Medical Faculty of the University of Cologne, and from the Laboratory for Autoimmune Diseases of the Wieslab Company, Sweden. Serum samples were taken from 304 AI patients, and 95 patients with GSD including untreated CD and DH patients (for numbers and diagnoses see Table IX). Sera from patients with psoriatic arthritis, hepatitis C, and different malignancies were also studied (Table IX). The term 'malignancies' includes two patients with primary liver carcinoma, three patients with thyroid gland carcinoma, and single cases with neuroblastoma, gastric carcinoma, hypernephroma, rectal adenocarcinoma, plasmocytoma, and a tumor of unknown origin giving metastases in liver, mediastinal lymphnodes, and bones. Twenty six of the 84 controls were healthy individuals while 58 had various non-AI diseases (Table X). Thus altogether 605 serum samples were analysed. The mean age and sex of the patient groups are presented in Table IX. All serum samples were stored at -78 °C until assayed.

Table IX. Age, sex, and median±95% CI of serum IgA Ab concentrations against the hTGc in controls and patients subdivided into diagnostic categories.

Diagnosis	**CD**	**DH**	**CTR**	**PV**	**BP**	**CU**	**GP**	**CR**
n	39	56	84	48	32	20	20	31
Mean age	17.5	33.9	19.4	51.9	67.0	48.3	72.8	41.4
Min.-max. age	1-66	6-74	6-56	9-85	39-92	28-76	49-97	15-99
Males/females	14/25	32/24	38/46	21/27	17/15	9/11	11/9	18/13
Median (AU)	89.5	63.5	12.1	13.2	14.1	20.9	23.8	20.9
95% CI (AU)	74.2-98.3	51.3-76.1	11.2-13.0	11.3-15.7	12.0-16.4	17.8-24.4	17.2-28.2	15.2-26.4

Diagnosis	**WG**	**RA**	**SLE**	**PSS**	**APS**	**PA**	**HC**	**TU**
n	20	41	49	30	13	11	100	11
Mean age	71.9	59.8	39.5	59.8	41.4	50.5	42.6	54.0
Min.-max. age	30-89	33-77	18-61	39-79	26-60	29-67	20-71	19-74
Males/females	12/8	2/15	1/24	13/17	4/8	5/6	58/42	6/5
Median (AU)	25.0	19.2	20.0	20.5	15.3	14.7	14.3	17.4
95% CI (AU)	19.9-32.7	16.1-22.6	15.0-25.2	17.5-23.6	13.3-19.0	12.1-20.6	13.5-15.3	12.2-36.6

Non-standard abbreviations see Fig. 19 on page 52.

The diagnoses of the AI diseases were confirmed by the following guidelines: CD: jejunal biopsy pathology and EMA-positivity (203, 283). DH, pemphigus vulgaris and bullous pemphigoid: conventional skin histology, direct and indirect immunofluorescence. Ulcerative colitis and Crohn disease: a combination of clinical, radiological, endoscopical, and histological signs. Goodpasture's syndrome: clinical symptoms together with immunofluorescence showing autoantibodies against glomerular basement membrane. Wegener's granulomatosis: 1990 American College of Rheumatology criteria; in addition, positivity for PR3-ANCA Abs (178). Rheumatoid arthritis, SLE and systemic sclerosis: American College of Rheumatology (former American Rheumatism Association) criteria (15, 263, 199). Primary antiphospholipid syndrome: clinical symptoms; at least one of the typical laboratory changes (positivity for lupus anticoagulant or anti-

cardiolipin Abs of IgG or IgM class) detected twice in a period of at least 8 weeks; the absence of any signs or symptoms of other AI disorders, infections, malignancies or adverse side effects of medicaments. Psoriatic arthritis: Wright and Moll's criteria (291). Hepatitis C: positive PCR analysis and/or positive serology together with clinical symptoms of liver disease including elevated transaminase levels and/or positive liver histology. Malignant tumors: histology.

Table X. Diagnoses and numbers of patients with non-AI diseases.

Diagnosis	No.
Anorexia nervosa	1
Cholelithiasis	1
Chronic pancreatitis	1
Cow's milk protein intolerance	3
Fructose intolerance	1
Gastroesophageal reflux	2
Giardiasis	2
Habitual constipation	1
Helminthiasis	2
Ichthyosis	17
Idiopathic chronic abdominal pain	1
Idiopathic chronic diarrhea	1
Infectious diarrhea	1
Irritable colon syndrome	1
Post-enteritis diarrhea	1
Primary adult lactose intolerance	10
Prolonged microcyter anemia	1
Psychosomatic diarrhea	1
Retarded growth (<3 percentile) due to impaired GI absorption of various origin	7
Retarded growth of other than GI origin	2
Urticaria	1

4.3.2. Total serum IgA measurement, gliadin ELISA and EMA test

The EMA test was performed with every serum from patients with CD or DH. In addition, the total serum IgA level was measured, and AGA and EMA tests performed on a randomized subgroup of AI patients having IgA Abs against hTGc. Those chosen suffered from the following disorders (number of patients in parentheses): SLE (17), rheumatoid arthritis (9), hepatitis C (15), psoriatic arthritis (3), progressive systemic sclerosis (19), primary antiphospholipid syndrome (4) and malignancy (3).

The total serum IgA was measured using the Tina-quant IgA kit (Roche Diagnostics GmbH, Mannheim, Germany), the IgA AGA were detected with the Bindazyme Antigliadin IgA kit (Binding Site Ltd, Birmingham, England) according to the instructions of the manufacturers. The total IgA was considered to be elevated when above 4.9 g/l in men and above 4.5 g/l in women, the cutoff of AGA ELISA was set according to the recommendations of the manufacturer. Serum IgA EMAs were measured as described previously (4.1.2).

4.3.3. TGc ELISAs

The hTGc and gpTGc ELISAs were set up and performed as described under (0 and 4.2.7). In this study, the same positive reference serum was used as previously (4.2.7), thus cutoff values of 14 and 18 AU were chosen for the guinea pig and the hTGc ELISAs, respectively.

4.3.4. Statistics

The description of statistical methods can be found under 4.2.8.

5. RESULTS

5.1. The guinea pig TGc study

ELISAs based upon the commercial gpTGc were derived and compared to the EMA test for diagnosis of GSD. Follow-up sera were studied to detect titre changes during a GFD, and inhibition of endomysial staining were performed with EMA-positive sera to study whether autoantigens other than TGc may be responsible for EMA positivity.

5.1.1. Guinea pig TGc ELISA

The mean intra- and interassay coefficients of variation of the positive standard serum were 2.5% and 9.1%, respectively. The mean intra- and interassay coefficients of variation (using serum titres given in arbitrary units) for the other sera were 4% ($n = 379$) and 14% ($n = 30$), respectively. The median Ab concentration was 55.0 AU (95% CI, 37.8-69.8 AU; $n = 67$) for the patients with untreated GSD (CD or DH), and 8.4 AU (95% CI, 8.1-8.7 AU; $n = 213$) for controls; the difference was significant ($P < 0.0001$). The median Ab concentration was 70.6 AU (95% CI, 57.0-85.6 AU; $n = 37$) for the patients with untreated CD, and 37.3 AU (95% CI, 26.3-53.3 AU; $n = 30$) for the patients with untreated DH; the difference was significant ($P = 0.0011$). The median Ab concentration was 10.5 AU (95% CI, 9.3-14.7 AU; $n = 88$) for GSD patients on GFD, 8.2 AU (95% CI, 6.8-75.3 AU; $n = 12$) for patients on GCH, 8.2 AU (95% CI, 7.8-8.7 AU; $n = 96$) for controls with GI diseases, and 8.5 AU (95% CI, 8.2-8.9 AU; $n = 117$) for healthy individuals and controls with other diagnoses. No significant difference was found between the Ab concentrations of these latter patient groups. The AUC was 0.979 (Fig. 12).

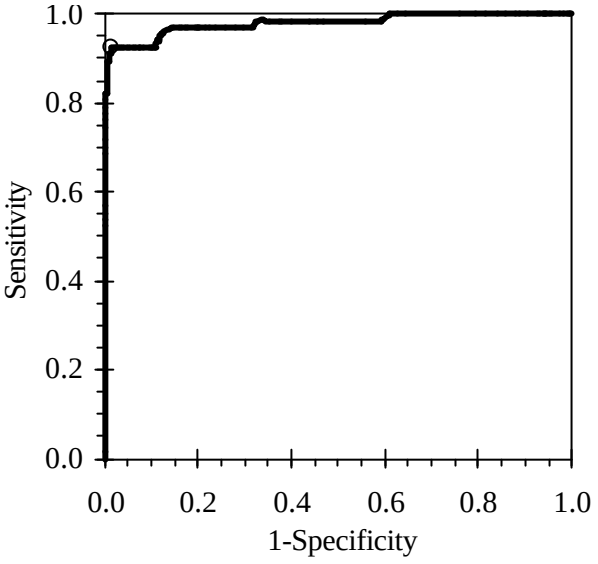

Fig. 12. The ROC curve for the gpTGc ELISA. The circle shows the point of greatest efficiency of the test upon which the cutoff level was chosen.

Based upon the ROC analysis, a cutoff value of 15.5 AU was chosen, and sera with Ab concentrations 15.5 AU were labeled as gpTGc ELISA positive. This cutoff value gave (excluding patients on a GFD or a GCH) a specificity and a sensitivity of 98.6% (95% CI, 97.2-100%) and 92.5% (95% CI, 89.5-95.6%), respectively. The coincidence of the results gpTGc assay with the clinical diagnoses (excluding treated patients) was 272 of 280 (97.1%), giving 3 false-positive and 5 false-negative results (Fig. 13).

One of the patients with false-positive sera had primary liver carcinoma, the other two patients had IgA-pemphigus; all the 3 patients were females. Three sera from pa-

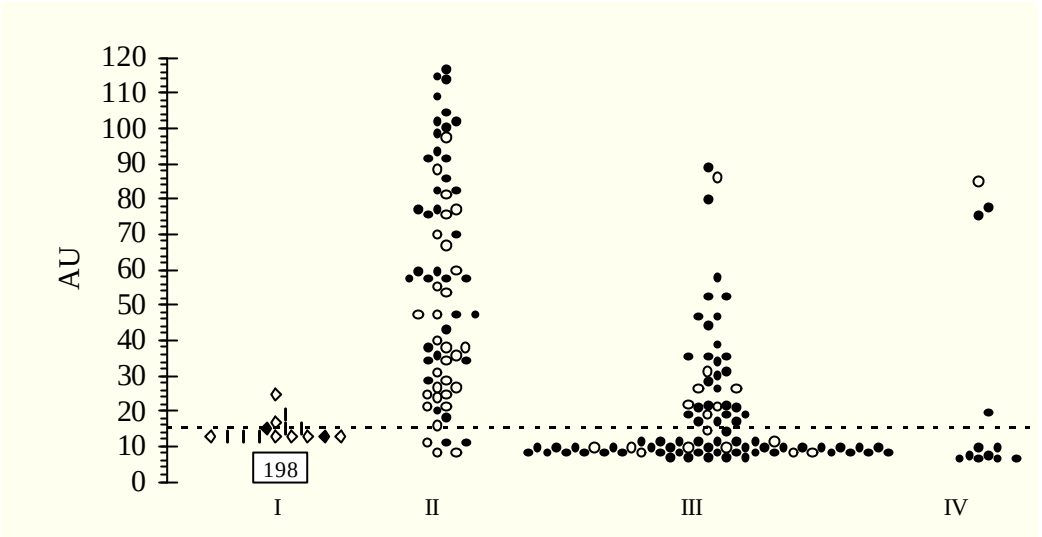

Fig. 13. Serum concentrations of IgA Abs against TGc in the gpTGc ELISA. The following patient groups are presented: I, controls (, with GI diseases; , with others); II, untreated patients; III, patients on a GFD; IV, patients on a GCH having CD ( ) or DH ( ). The box in the control group represents 198 individuals with a serum concentration of IgA Abs against TGc less than 12.4 AU. The chosen arbitrary cutoff level for positivity (dashed line) is drawn at the AU of 15.5.

tients with CD and two samples from patients with DH were false-negative, the latter two false-negative samples were also negative for EMAs.

All of the 13 follow-up sera were EMA-positive and had elevated serum IgA Ab titres against the gpTGc before the introduction of a GFD. Changes during the GFD are presented in Fig. 14. With the exception of two cases, a reduction of the titres could be seen on the GFD, even if in the vast majority of the cases the result of the EMA test did not change. In one case, an 11-year-old girl with CD showed elevation of titres after 5 months which was correlated with gliadin consumption, and following a strict dietary regimen, the titres fell to normal after a year (data not shown). In a male with DH, after the EMA test had become negative, EMA positivity was detected again in parallel with elevation of IgA Ab titres against TGc indicating dietary failure.

5.1.2. Comparison of the EMA test with the guinea pig TGc ELISA

The results of the EMA and gpTGc ELISA tests coincided in 364 of 380 cases (95.8%). The EMA test gave a false-negative result for 2 sera from untreated DH patients, these sera were also negative in the TGc ELISA.

Eleven of 109 EMA-positive samples (10.1%) were false-negative in the TGc ELISA including also sera from patients on a GFD or a GCH. One of the false-negative samples was from an untreated DH patient, two from untreated CD patients, and the rest from CD patients on a GFD.

TGc ELISA positivity with EMA negativity was detected in two sera from patients with GSD, one on a GFD (21.5 AU), the other on a GCH (19.5 AU). The three false-positive sera with TGc ELISA from control patients were diagnosed as negative with the EMA test. Compared to the EMA results, including only untreated GSD patients and using the same cutoff level, the specificity and sensitivity of the gpTGc ELISA method was 98.6% (95% CI, 97.2-100%) and 95.4% (95% CI, 93.0-97.9%), respectively. In-

cluding also patients on a GFD or a GCH, the sensitivity of the gpTGc ELISA was lower (89.9%; 95% CI, 86.9-92.9%).

5.1.3. EMA inhibition

Nine sera with EMA positivity and serum IgA Ab activity against TGc lower than 11.5 AU were used. One of the samples was from an untreated CD patient, another one from an untreated DH patient, and the rest (7 sera) from CD patients on a GFD. The evaluation was difficult because TGc alone gave non-specific fluorescence on the esophagus sections resulting in higher background. In four samples, the endomysial immunofluorescence could be abolished. In four cases, a significant reduction of the signal could be detected, but the high background did not allow the differentiation between complete absence of the signal or its reduction with some residual immunofluorescence. One sample from a CD patient on a GFD with a very low Ab titre (6.7 AU) remained positive.

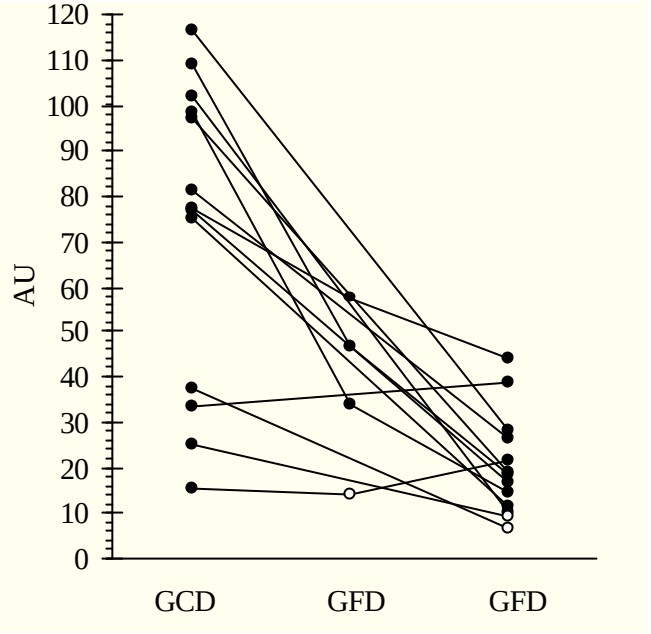

Fig. 14. Changes of serum IgA antibody titres against TGc in 13 patients after introduction of a GFD (GCD, gluten containing diet). The lines connect points representing titre values from the same patient. EMA-negative samples are shown by empty circles, EMA-positives by full points. Serum samples were taken at different time points after introduction of the GFD. Serum titres drop even if the EMA results are still positive, which can be useful in clinical practice by showing patient compliance.

5.2. The human TGc study

To test whether the performance of the TGc ELISA could be improved, recombinant human TGc was produced and used as the basis of an ELISA. This was compared to results from a gpTGc based assay and EMA tests on monkey oesophagus.

5.2.1. Recombinant human TGc

The hTGc was expressed in the 293-EBNA human embryonic kidney cell line as a fusion protein with the Strep II tag. The protein could be purified in a single step by affinity binding to a StrepTactin coloumn; on washing with desthiobiotin, the protein eluted as a single band with an estimated molecular mass of 87 kDa (Fig. 15A) when visualised by Coomassie-stained SDS-PAGE. Immunoblot analysis showed that the band reacted with MAbs against TGc (Fig. 15B). The coloumn bound almost all the tagged protein with no immunoreactivity appearing in the flow through (Fig. 15B). The yield from the lysate of a confluent cell monolayer in a cell culture dish of 13 cm diameter was ~200 ìg. The molecular mass calculated from the sequence of the hTGc is

ameter was ~200 ìg. The molecular mass calculated from the sequence of the hTGc is 77.3 kDa, and the calculated molecular mass of the fusion protein (TGc having a C-terminal tag of 10 amino acids) is 78.4 kDa. Mass spectrometry of the fusion protein gave a molecular mass of 78.3 kDa. In cell lysates, the activity of the expressed human TGc was 4.7-fold higher than the background activity of TGs present in untransfected 293-EBNA cells. The freshly purified hTGc showed similar or higher activity than the gpTGc from Sigma, but it lost activity on storage. It is not known whether the hTGc has the same catalytic activity as the guinea pig enzyme.

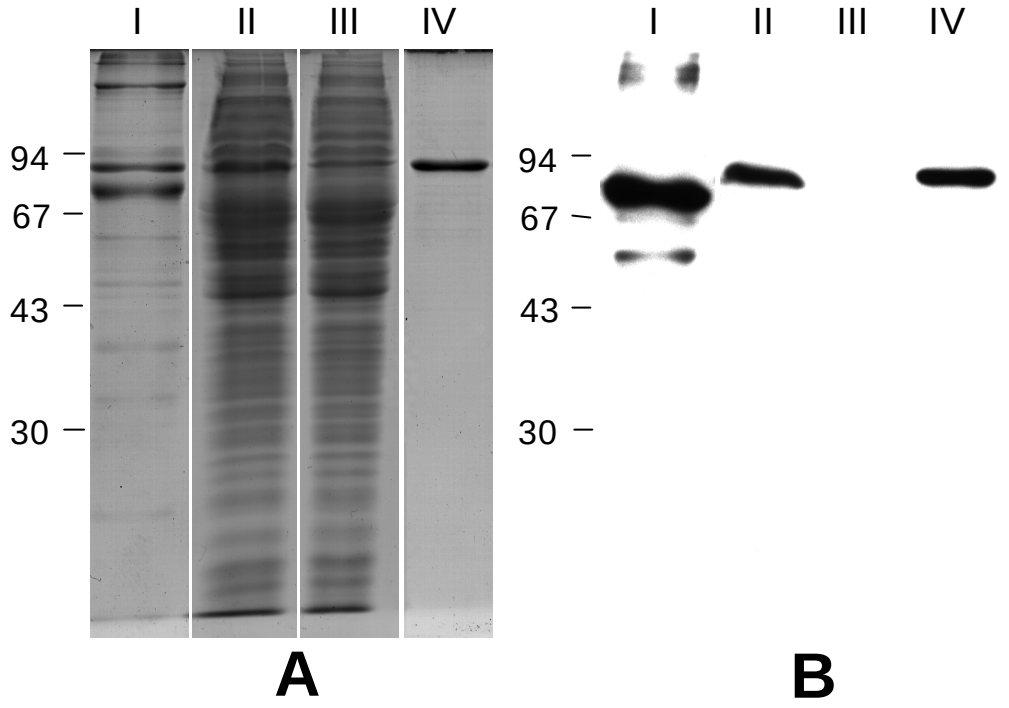

Fig. 15. SDS-PAGE (A) and immunoblot (B) analysis of TGc. The immunoblot was performed using monoclonal antibodes against TGc. Positions of molecular mass standards (kDa) are indicated on the left. Lane I, guinea pig TGc; lane II, lysate of cells producing recombinant human TGc before purification; lane III, flow through; lane IV, eluted TGc from the column.

5.2.2. Performance of the human TGc ELISA

The optimal coating concentration of hTGc was 1 ìg per well. Using highly positive sera from four patients for calibration, we obtained a log-linear curve between dilutions of 1:250 and 1:32,000. Four negative sera showed some signal at lower dilutions (>1:500). Some positive sera showed a signal plateau at dilutions of 1:250 or less. The ratio between the mean absorbance values of positive and negative results at the dilution of 1:125 was 1:6, whereas at higher dilutions, it was >1:10. Hence, in the assay a serum dilution of 1:250 was used. One positive and one negative reference serum sample was included in each assay to control the test performance. The positive serum was used as the 'standard', and the absorbance results were given as AU, calculated as a percentage of the standard serum. The mean intra- and interassay coefficients of variation for the positive standard serum were 1.3% and 13.7%, respectively. The mean intra- and inter-

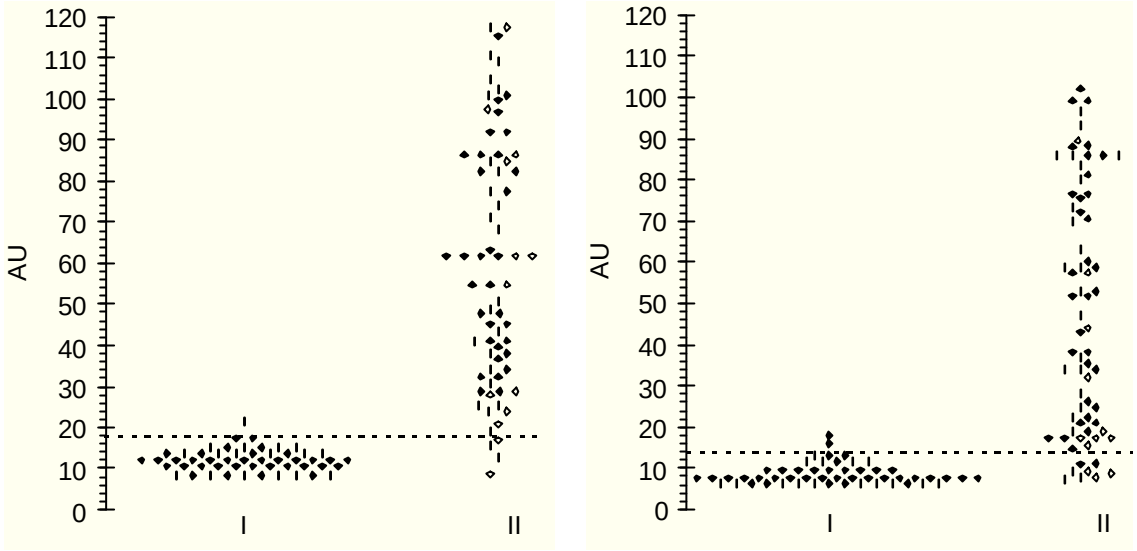

Fig. 16. Serum concentrations of IgA antibodies against TGc in controls (I) and in patients having CD or DH (II; , untreated; , treated CD or DH patients). **A**, the hTGc ELISA. **B**, the guinea pig TGc ELISA. The chosen arbitrary cut-off level for positivity (broken line) is drawn at the AU of 18 or 14, respectively. The ROC curves are shown as insets.

assay coefficients of variation (using serum titres given in AUs) for the other sera tested in the hTGc ELISA were 3.2% ($n = 124$) and 9.2% ($n = 15$), respectively.

The median Ab concentration was 61.4 AU (95% CI, 45.1-78.5 AU; $n = 55$), for patients with untreated GSD (CD or DH) and 12 AU (95% CI, 10.8-13 AU; $n = 53$) for controls; the difference was significant ($P < 0.0001$). The median Ab concentrations were 74.2 AU (95% CI, 47.7-86.7 AU; n=25) and 49.4 AU (95% CI, 39.7-63.0 AU; n=30) for untreated patients with CD and DH, respectively; the difference was not significant (P=0.1142). The median Ab concentration was 48.1 AU (95% CI, 20.8-85.6 AU; $n = 16$) for treated patients, 12.1 AU (95% CI, 9.8-14.7 AU; $n = 26$) for controls with gastrointestinal diseases, and 12 AU (95% CI, 10.7-13.0 AU; $n = 27$) for healthy individuals and controls with other diagnoses. The AUC was 0.999 (95% CI, 0.996-1.001; 95% CI with BC_a method, 0.990-1.0; Fig. 16A).

A cutoff value of 18 AU was chosen, and sera with Ab concentrations 18 AU were labelled as hTGc ELISA positive. This cutoff value gave a specificity and a sensitivity of 98.1% (95% CI, 95.7-100%) and 98.2% (95% CI, 95.9-100%), respectively (treated patients were excluded). The coincidence of the hTGc assay with the clinical diagnosis (excluding treated patients) was 106 of 108 (98.1%), giving one false-positive and one false-negative result (Fig. 16A).

5.2.3. Performance of the guinea pig TGc ELISA

The optimal coating concentration of gpTGc was 1 ìg per well, and the optimal serum dilution 1:250, as with the hTGc ELISA. Each assay was performed parallel to the hTGc assay at the same time, and the same serum samples and serum dilutions were used. The mean intra- and interassay coefficients of variation of the positive standard serum were 2.2% and 9.0%, respectively. The intra- and interassay coefficients of variation (using serum titres given in AUs) for the other sera tested in the gpTGc ELISA were 2.8% ($n = 124$) and 12.8% ($n = 15$), respectively.

The median Ab concentration was 51.8 AU (95% CI, 34.2-63 AU; $n = 55$) for the patients with untreated GSD (CD or DH), and 8 AU (95% CI, 7.3-8.9 AU; $n = 53$) for controls; the difference was significant ($P < 0.0001$). The median Ab concentration was 18 AU (95% CI, 9.2-69.9 AU; $n = 16$) for treated patients, 7.5 AU (95% CI, 6.6-9 AU; $n = 26$) for controls with GI diseases, and 8.5 AU (95% CI, 7.2-10.3 AU; $n = 27$) for healthy individuals and controls with other diagnoses. The AUC was 0.980 (95% CI, 0.958-1.002; 95% CI with BC_a method, 0.943-0.993; Fig. 16B).

A cutoff value of 14 AU was chosen, and sera with Ab concentrations 14 AU were labelled as gpTGc ELISA positive. This cutoff value gave (excluding treated patients) a specificity and a sensitivity of 96.2% (95% CI, 92.8-99.6%) and 92.7% (95% CI, 88.1-97.3%), respectively. The coincidence of the gpTGc assay with the clinical diagnosis (excluding treated patients) was 102 of 108 (94.4%), giving 2 false-positive and 4 false-negative results (Fig. 16B).

5.2.4. Effects of Ca^{2+}-activation

Thirty-two serum samples were tested in the ELISA for IgA Abs against hTGc with and without calcium-activation. The overall Ab titres did not show a significant difference ($P = 0.27$). However, sera with anti-TGc titres <30 AU in the calcium-activated assay were significantly lower in the assay without calcium activation ($n = 18$; $P = 0.009$), whereas higher titres were not significantly different ($n = 14$; $P = 0.35$).

5.2.5. Comparison of EMA test with TGc ELISA

Excluding treated patients, with the exception of one false-positive result, all of the patients with EMA-positive sera had GSD (55 of 56, 98.2%). Twelve of 16 (75%) treated patients with GSD were positive for EMAs. Comparing only the untreated EMA-positive cases, the results of human and gpTGc ELISAs coincided with the EMA test in 54 of 56 (96.4%) and 51 of 56 (91.1%) cases, respectively. The serum that gave a false-positive result in the EMA test was negative in both the human and the gpTGc ELISAs. The one serum that gave a false-negative result in the hTGc ELISA was also negative in the gpTGc ELISA. The 12 patients on incomplete GFDs with EMA positivity had also positive anti-TGc IgA titres with both ELISA systems.

All patients negative for EMAs were either treated patients having GSD or patients not having GSD. Comparing only the untreated EMA-negative cases, the results of the human and gpTGc ELISA coincided with the EMA test in 51 of 52 (98.1%) and 50 of 52 (96.2%) cases, respectively. The one serum that gave a false-positive result in the hTGc ELISA was also positive in the gpTGc ELISA; in addition, another serum that gave a false-positive result was detected by the guinea pig assay. Both false-positive sera were from patients with Crohn disease. The four EMA-negative patients with treated GSD were also negative by gpTGc ELISA, but one of them was positive by hTGc ELISA.

The overall coincidence of the EMA test with human and gpTGc ELISA was 120 of 124 (96.8%) and 117 of 124 (94.4%), respectively.

5.2.6. Comparison of human TGc ELISA with guinea pig TGc ELISA

The results of the two ELISAs coincided in 119 of 124 (96%) of all tested sera. In four discordant cases, the human assay was more sensitive than the guinea pig assay, giving positivity in the human assay. One of the sera was from an EMA-negative CD patient on a gluten-free diet. In the fifth discordant case, the gpTGc ELISA gave a false-

positive result for a patient with Crohn disease. The Ab titre, however, was also high (17.5 AU) in the hTGc ELISA, almost reaching the cutoff value (18 AU).

The false results of the hTGc ELISA coincided with those of the gpTGc ELISA. Both assays failed to recognise the serum of one EMA-positive CD patient, and both detected a patient having Crohn disease as positive. Both tests gave a correct, negative result in the case of a false EMA-positive patient.

The titres obtained with the two assay correlated well ($r_S = 0.9377$; 95% CI, 0.9121-0.9559; $P < 0.0001$); the correlation was theoretically exponential, but in practice it was linear, with an exponent of 1.05 (Fig. 17). The difference between the AUCs was 0.019 (95% CI, -0.002 to 0.040; 95% CI with BC_a method, 0.005-0.056).

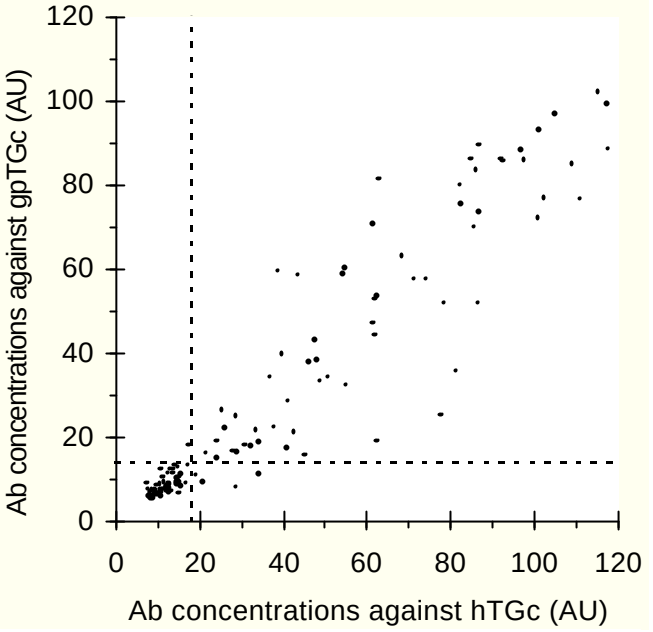

Fig. 17. Correlation of Ab titres between the human and the gpTGc ELISA. *x*-axis, titres given in AUs measured by the hTGc ELISA; *y*-axis, titres measured by the gpTGc ELISA. The cutoff values are presented by dashed lines.

5.3. The autoimmune study

The specificity of the TGc ELISAs was tested using serum samples from patients having a wide spectrum of different diseases, in particular AI patients without GSD and patients with diseases involving enhanced apoptosis and cell lysis, where release of TGc could theoretically lead to anti-TGc Ab formation.

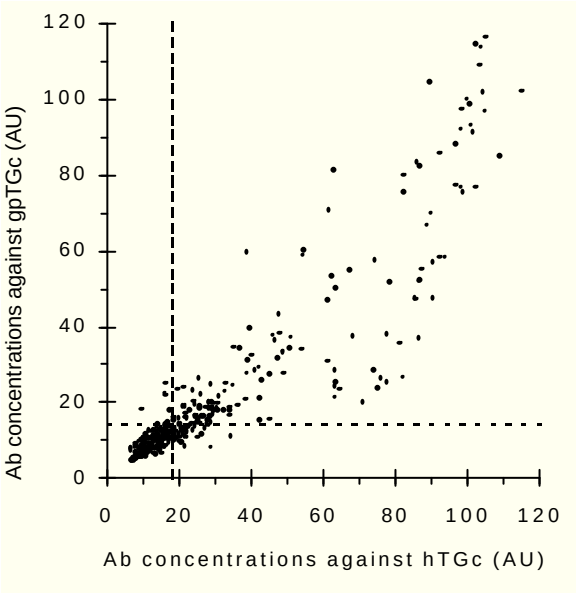

Fig. 18. Correlation of Ab titres between the human and the gpTGc ELISA in the AI study. *x*-axis, titres given in AUs measured by the hTGc ELISA; *y*-axis, titres measured by the gpTGc ELISA. The cutoff values are presented by dashed lines.

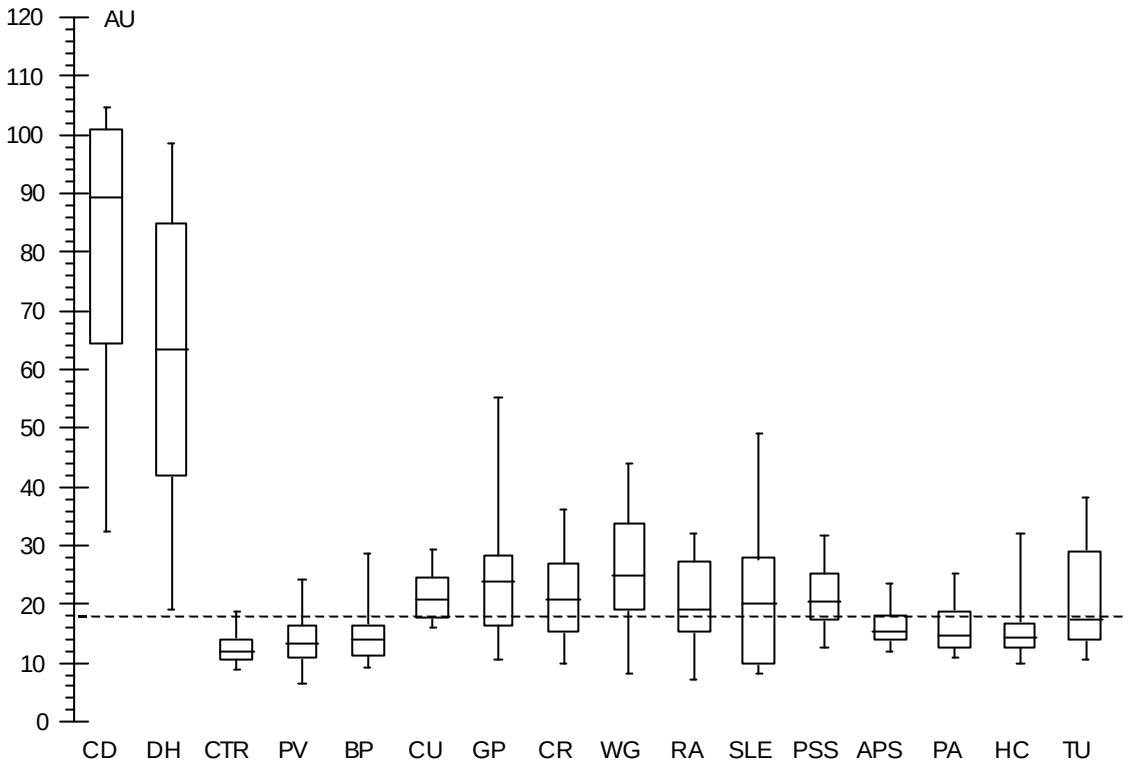

Fig. 19. Box and whisker diagram showing the serum IgA Ab concentrations against the hTGc in controls and patients subdivided into diagnostic categories. The lower and upper edges of the boxes represent the 25% and 75% percentiles, respectively. The median is indicated by a horizontal line through the box. Exact values and 95% CIs see in Table IX on page 43. The lower and upper whiskers represent the 5% and 95% percentiles, respectively. The arbitrary cutoff level for positivity is drawn by dashed line at the AU of 18. **Non-standard abbreviations:** CTR, controls; PV, pemphigus vulgaris; BP, bullous pemphigoid; CU, ulcerative colitis; GP, Goodpasture's syndrome; CR, Crohn disease; WG, Wegener's granulomatosis; RA, rheumatoid arthritis; PSS, progressive systemic sclerosis; APS, antiphospholipid syndrome; PA, psoriatic arthritis; HC, hepatitis C; TU, malignant tumor.

5.3.1. TGc ELISA

The results from the human and gpTGc ELISAs correlated strongly ($n = 379$; $r_S = 0.91703$; 95% CI, 0.8994-0.9317; $P < 0.0001$) (Fig. 18). Thus, as the hTGc ELISA was shown previously to be the superior (5.2.6), only the results from this test are presented here.

The median serum Ab concentrations against the hTGc are shown in Fig. 19 and Table IX on page 43. The difference between the two forms of GSD was significant ($P = 0.0004$), the 95% CIs, however, overlapped. One (EMA-positive) patient with CD and two (EMA-negative) patients with DH fell below the cutoff of 18 AU in the hTGc ELISA. No healthy individual had TGc Ab levels greater than the cutoff; however, five control patients had Ab concentrations just greater, in the range of 18.5-22.1 AU. These had been diagnosed as having (number of patients in parentheses) ichthyosis (3), retarded growth of non-gastrointestinal origin (1), primary adult lactose intolerance (1).

Comparing CD and DH patients with the controls, the sensitivity and specificity of the hTGc ELISA was 96.8% and 94.0%, respectively.

Overall 49% of all non-GSD AI sera were positive in the hTGc ELISA (somewhat fewer, 39.4% were positive in the gpTGc ELISA). In some individual diagnostic groups there were low median levels with elevated titres only in a few patients (e.g. bullous pemphigoid and pemphigus vulgaris), whereas other groups had high median Ab levels (e.g. Wegener's granulomatosis and Goodpasture's syndrome) (Fig. 19 and Table IX on page 43). The difference between the median titres of the AI and the control sera was significant in every case except for the pemphigus vulgaris sera (bullous pemphigoid, $P = 0.012$; pemphigus vulgaris, $P = 0.152$; SLE, $P = 0.0005$; antiphospholipid syndrome, $P = 0.0002$; for each of the other groups $P < 0.0001$).

Significant differences were also found in serum titres between controls and patients with hepatitis C ($P < 0.0001$), psoriatic arthritis ($P = 0.0065$), and malignancies ($P = 0.0005$) when compared by the Mann-Whitney test. Altogether 25% of these patient groups were positive in the hTGc ELISA. While in most of the disease groups the 95% CIs confirmed the difference to the controls as being significant, the 95% CIs overlapped in three (bullous pemphigoid, psoriatic arthritis, and malignancies), indicating that further studies are needed with patients suffering from these diseases.

5.3.2. Total serum IgA, AGA ELISA and EMA test

Of the non-GSD patients with TGc serum Ab levels above the cutoff, 70 were chosen at random for the measurement of total serum IgA levels, these sera were also tested for AGA and EMA reactivity. Fifteen (21.4%) had elevated total IgA levels, although one was borderline (Table XI). Increased total IgA levels occurred in most diagnostic groups, but it is of note that it was not seen in any patient with primary antiphospholipid syndrome; on the other hand it was most common in patients with rheumatoid arthritis (4/9) and malignancies (2/3) (Table XI). No patient had IgA-deficiency.

AGA-positivity was found in 8 of the 70 patients (11.4%), one or two AGA-positive sera being found in each diagnostic group, again with the exception of patients with primary antiphospholipid syndrome. Five of these 8 sera also had an elevated total IgA. The correlation of total IgA levels with the AGA concentrations was significant; however, there was a large degree of scatter ($P < 0.0001$; $r_S = 0.6451$; 95% CI, 0.4832-0.7641; Fig. 20A).

Table XI. Frequency of elevated total IgA level as well as AGA and EMA positivity of AI sera positive for hTGc ELISA.

	RA	SLE	PSS	APS	PA	HC	TU
No. tested	9	17	19	4	3	15	3
Elevated total IgA	4	2	2	0	1	4	2
AGA	1	1	2	0	1	2	1
EMA	0	0	0	0	0	0	0

Abbreviations: see Fig. 19.

None of the sera was positive for EMA. However, some sera from patients with SLE and hepatitis C showed signals on the monkey esophagus sections arising from nuclei, epithelial pericellular and/or reticular structures different from the endomysium. Twelve of the 15 sera with elevated total IgA had markedly elevated IgA Ab concentrations against the hTGc (above 25 AU), and 58.8% of the sera giving over 30 AU in the hTGc ELISA had elevated total IgA. The hTGc ELISA results correlated significantly with the total IgA level ($P = 0.0026$), but the scatter of the data was high ($r_S = 0.3544$; 95% CI, 0.1303-0.5441; Fig. 20B). The same was true for the correla-

tion of the hTGc ELISA results with the AGA concentrations ($P = 0.0034$; $r_S = 0.3454$; 95% CI, 0.1201-0.5367; Fig. 20A).

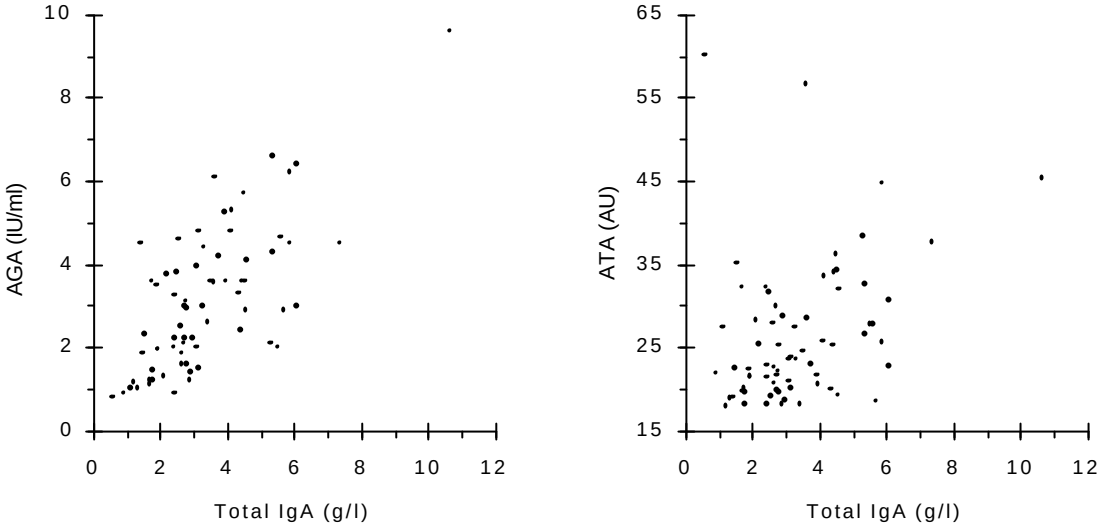

Fig. 20. Correlation of the total IgA level **A**, with the IgA Ab concentration against gliadin (AGA); **B**, with the IgA Ab concentration against hTGc (ATA). Note the relatively high scatter of the values.

6. DISCUSSION

6.1. Diagnosis of patients and controls

GSD patients were diagnosed in all the three studies according to current criteria (283, 276); however, GSD was not excluded histologically in all controls. In particular, this may be a problem in the interpretation of the results of the AI study. GSD is expected to be very uncommon without GI symptoms typical of GSE, with the symptoms of another disease, and negative EMA test. In these patients, it is very rare to find histological changes showing evidence for the presence of GSE; the probability of an indisputably positive histological result is approximately 0.5% (32). As the risk, particularly in children, of an invasive biopsy procedure outweighs that of a misdiagnosed silent CD, such biopsies were not carried out. Further, as there is little evidence that symptomless and EMA-negative CD has to be treated, we beleive a positive TGc ELISA result alone does not provide ethical grounds for performing an intestinal and/or a cutaneous biopsy.

In conclusion, though some of the 'false-positive' controls could have silent CD, the expected frequency of EMA-negative GSD (estimated to be lower than 1-2%) is far below the limit above which it could cause significant changes in the interpretation of results and thus in the message of the studies.

6.2. TGc preparations

6.2.1. Expression of active, recombinant human TGc.

To our knowledge, recombinant hTGc had not been expressed before in mammalian cells. We preferred using human cells instead of bacteria for two reasons. First, although there is no evidence for posttranslational modifications of TGc, we cannot completely exclude this possibility. Such modifications would in all probability not occur in bacteria. Second, if chaperons are needed to obtain a correct folding, these are more likely to be present in human cells.

We decided to use a tag polypeptide to enable quick and efficient purification of hTGc. For this purpose, we used the Strep II tag for the following reasons:

1. The Strep-tag II consists of only 8 amino acids and so is unlikely to influence the features and functions of proteins.

2. It can be used both N- and C-terminally. Theoretically, even combination with a signal peptide is possible (TGc is known not to have a signal peptide).

3. It can, if required, be cleaved after purification, the cleaved products can be easily separated from the authentic recombinant protein using biotinylated thrombin (factor Xa). (However, we did not cleave it in our studies.)

4. Simple, one step purification is possible. Preliminary studies showed that the StrepTactin coloumn works extremely efficiently. In addition, the purification of reasonable amounts of TGc from cell lysate can be carried out within 6 hours. During affinity chromatography, physiological conditions can be used (no need for high salt con-

centrations, extreme pH, reducing or chelating agents), but the use of such conditions is theoretically feasible. Thus mild elution without loss of function and/or folding can almost always be done. In the case of TG purification, EDTA should be added during purification for inhibition of enzyme activity. This would not have been possible if the His-tag had been used, because addition of EDTA during affinity purification of His-tagged proteins destroys the coloumn matrix. The eluting agent (desthiobiotin) is a vitamin derivate which can be easily removed by dialysis or gel chromatography if necessary (it was not removed in our studies).

5. There is a very sensitive and specific polyclonal Ab against the tag which makes detection of expression easy.

6. The affinity coloumn can be reused several times making the system economic. The costs for the whole hTGc purification and detection system were below $300.

EBNA cells were used as the expression system as they usually produce high yields of the recombinant protein after episomal transfection with CMV promotored vectors, are easily transfected and do not show contact inhibition.

The molecular mass of the gpTGc differs only slightly (0.1 kDa) from that of the hTGc when measured by mass spectrometry, but the gpTGc migrates appreciably faster on SDS-PAGE than the hTGc. Although the molecular mass difference is 1.2 kDa, the TGc from human fibroblasts migrates with the same speed as our fusion protein implying that the difference between the structures of the human and the gpTGc is more profound than suggested by their high amino acid identity.

6.2.2. The guinea pig TGc.

The guinea pig liver TGc preparation from Sigma contains other protein contaminants which are not immunoreactive with MAbs against TGc (Fig. 15). However, as it had been used successfully in other studies (69, 71, 259), we did not purify it further. It cannot be excluded that immunopositivity seen might in some cases be due to reactivity against contaminants.

6.2.3. Effects of Ca^{2+}-ions

'Ca^{2+}-activation' of TGc should cause conformational changes and has been used to optimise the TG assay systems (71, 259). The avidity of CD autoantibodies to TGc is known to be very low to the denatured form (69), thus its conformation is of great importance. In our assays, the sensitivity of the Ca^{2+}-activated hTGc ELISA was also higher than that without Ca^{2+}-activation; however, the role of Ca^{2+} in the binding of Abs in GSD sera has generated some controversy (e.g. 259 and 71 versus 270 and 215).

It has been shown that IgA autoantibodies bind significantly less to TGc conformations other than the Ca^{2+}-activated form even if the Ab-TGc reaction itself occurs in a Ca^{2+}-deficient buffer (162). Possibly these conformational changes in the structure of TGc reveal epitopes characteristic of the extracellular form. It is also possible that activated TGc may deamidate itself producing neoepitopes against which higher affinity Abs might be directed. Finally, while Ca^{2+}-activated TGc could theoretically autocross-link itself, recent work suggests that this does not result in significant increase of the amounts of TGc bound to the ELISA well surface (162).

The effect of Ca^{2+} could also be seen using the anti-TGc MAb TG100 (Neomarkers, presumably having a binding site in barrel 1 near the Ca^{2+}-binding region of TGc between aa 447-538), whereas no difference could be observed using another anti-TGc MAb (CUB 7402, Neomarkers, presumably having a binding site in barrel 1 near the Ca^{2+}-binding region of TGc between aa 447-478, spatially near the GTP-binding region). Although these MAbs always perfectly stain extracellular TGc (163), do not constantly recognise intracellular TGc (163, 239, Sárdy et al., unpublished observations) suggesting the epitopes of intracellular TGc are hidden in certain cases due to the absent Ca^{2+}-activation and/or to allosteric inhibiton by a bound intracellular component not present in the extracellular space. Indeed, an intracellular binding partner of TGc (calreticulin, the $G\hat{a}_h$ subunit, see 2.3.3.3.2) is known to inhibit the binding of GSD sera or the Neomarkers' MAbs to TGc (162). Autoantibodies from GSD sera are beleived to have cystein molecule(s) in their epitopes, because irreversible inhibitors of TGc (e.g. cystamine) targeting the -SH groups of cysteins (including the Cys^{277} in the catalytic centre) inhibit their binding to TGc (162). Ca^{2+}-ions can profoundly alter the accessibility of Cys residues as indicated in S-nitrosylation experiments (174), and preliminary plasmon surface resonance results indicate that the binding of GSD Abs to TGc has different kinetics in the presence or absence of Ca^{2+} (162).

Together these results indicate a role of Ca^{2+} ions in causing an irreversible conformational change altering the reactivity of IgA Abs in GSD to TGc, and support the notion that intracellular TGc has a conformation and/or binding partners making it unreactive to GSD sera.

6.3. Sensitivity and specificity

6.3.1. Setting the cutoff values

Setting of the cutoff values for the ELISAs was based on the ROC-analysis of the tests. A cutoff value providing perfect separation of individuals with or without GSD could not be found, though the coincidence with the diagnosis by biopsy was very high in both tests. The cutoff of the gpTGc ELISA was different in the two studies (14 and 15.5 AU in the gpTGc and hTGc ELISA study, respectively). This difference may be due to the different sets of sera used in the two studies, and it is within an expected range of measurement error. As both the false-negative and the false-positive values usually lie near the cutoffs, it would be feasible to determine 'gray zones' in both assays (14-20 AU for the gpTGc and 16-22 AU for the hTGc ELISA) in which values should be regarded questionable. EMA-negative patients having anti-TGc Ab concentrations in these zones should be retested after a few months gluten challenge, but an invasive examination is not necessary if there are no other symptoms or signs suggesting GSD. On the other hand, EMA-positive patients in these zones should be submitted to intestinal biopsy.

In the AI study, it was very difficult to judge whether the cutoff values were appropriate. Consequently, the current gpTGc and hTGc ELISA methods are not the methods of choice for serological diagnosis of GSD in patients having AI diseases other than GSD.

6.3.2. Sensitivity and specificity

6.3.2.1. The guinea pig TGc ELISA study

The gpTGc ELISA proved to be a suitable method for the diagnosis of untreated GSE, even though both some false-negative and false-positive results were observed. False positivity of the gpTGc ELISA could be observed in three serum samples. Repeated tests gave the same results. These patients suffered from IgA-pemphigus and primary liver carcinoma. None had clinical signs or symptoms of CD. Upon this result, one of the adult patients with IgA-pemphigus agreed to have a jejunal biopsy performed, but the histology was negative for GSE. The patient with liver carcinoma died before the beginning of this study, and jejunal histology was not performed. Biopsy of the third patient with IgA-pemphigus, an 80-year-old woman, was not considered because the possible diagnosis of silent CD would not have had therapeutic consequences. We could not find any obvious connection between these patients. It is conceivable that in the cases of IgA-pemphigus the result was caused by unspecifically bound IgA due to high IgA Ab concentrations against autoantigens other than TGc. The patient with liver carcinoma might have had silent CD, but it is also possible that she had autoantibodies against liver proteins being present in the gpTGc preparation.

The gpTGc ELISA was not as sensitive as the EMA. The lower sensitivity possibly was due to the use of the heterologous protein [the aa sequence identity between gpTGc and hTGc being only 82.8% (104, 133)], as four of the five false-negative sera were recognised as weakly positive in the hTGc ELISA. However, the endomysial binding of two of these four sera could be abolished by addition of excess gpTGc, suggesting the IgA Abs of low titre in these sera are most likely directed against epitopes of hTGc which are only partially conserved in the gpTGc, and so give weaker binding to the guinea pig antigen.

Although a few authors suggested TGc being a major but not the only antigen in monkey esophagus sections (185, 271), there is strong evidence for TGc being the only endomysial, reticulin, and jejunal autoantigen (162). This is why the lower sensitivity of gpTGc ELISA compared to the EMA test can be explained by the conformational differences between the guinea pig and the monkey TGc rather than by the presence of additional autoantigens in the oesophagus tissues.

Interestingly, among the 12 EMA-negative controls with serum IgA Ab titres below the cutoff but higher than 12.4 AU (see group I in Fig. 13) there were four direct relatives of CD patients. Repeated tests from serum samples taken at a later time point also showed EMA negativity and similar titres. In the absence of clinical signs of CD, intestinal biopsies were not performed (see 6.1), but this group of individuals will be further studied.

6.3.2.2. The human TGc ELISA study

The hTGc ELISA was found to be slightly superior to the gpTGc ELISA. The 95% CIs of sensitivities of the two ELISAs overlap; therefore, the sensitivity difference must be confirmed by further studies, but the results affirm the assumption that in a few cases Abs are directed against epitopes of hTGc not (or only partially) conserved in gpTGc.

The EMA test gave a false-positive result in an 8-year-old girl who had a transient diarrhoea in February 1998. Repeated EMA tests showed IgA binding in the intercellular spaces of smooth muscle cells. Jejunal histology was negative for GSE, and the diarrhoea has not recurred. The serum was not immunoreactive in either ELISA, and the

titre values were so far below the cutoff that the results were probably not random. The fact that a serum was false-positive by EMA test [which is very rare (22, 47)], but correctly diagnosed by both ELISAs, underlines the possibility of EMA positivity due to antigens other than TGc.

It was interesting that two EMA-negative patients with Crohn disease had TGc Ab titres above the cutoff level in the gpTGc ELISA, one of them also in the hTGc ELISA. Coeliac disease and Crohn disease have been described to occur in the same patient (107), but this association is very rare. In our two cases associated CD cannot be ruled out, but as both titres were near to the borderline (21.6 and 17.5 AU in the hTGc ELISA, 15.9 and 17.8 AU in the gpTGc ELISA), the elevation of titres might result from a low level IgA autoantibody production against TGc in Crohn disease rather than from that in active coeliac disease. Aspecific detection of IgA autoantibodies directed against minor contaminants in the TGc preparations is also conceivable. Indeed, in both ELISA systems the median of titres of patients with Crohn disease was greater than that of healthy individuals and patients with other gastrointestinal or non-gastrointestinal diseases. However, the differences and the number of sera tested in the hTGc ELISA study were too small to allow us to judge the significance of this finding.

6.3.2.3.The autoimmune study

In this study, almost half of the patients with non-GSD AI disease had appreciable IgA Ab levels in the hTGc ELISA system, whereas healthy controls were negative. The definitive identification of GSE requires small intestinal biopsy, but as detailed above (6.1), this was performed on ethical grounds only in patients whose diagnosis required this procedure. While a small percentage of the patients diagnosed as having non-GSD AI disease together with increased serum levels of anti-TGc IgA Abs possibly had silent CD, it is highly unlikely that such high numbers, particularly in the absence of EMA immunoreactivity, would have GSD.

The TGc ELISA cutoff was set at 18 AU, resulting in a sensitivity of 96.8% and a specificity of 94%. Increasing the cutoff to 25 AU, the specificity becomes 100% with respect to the control group, and also all patients with psoriatic arthritis and primary antiphospholipid syndrome fall below the cutoff value. However, even with this cutoff, 25% of all AI sera can still be considered to have raised serum levels of IgA Abs against TGc.

CD has been described to associate with various AI disorders (Table XII; see 274, 59, 130, 63, 143, 211, 55, 56, 227, 278, and references therein). While some of these associations are proven [e.g. AI thyroid diseases (274) and type I diabetes mellitus (59)], others are anecdotal.

Table XII. AI diseases having been suggested to be associated with GSD.

Addison's disease
AI hemolytic anemia
AI myocarditis
AI thrombocytopenic purpura
AI thyroid diseases
Atrophic gastritis – pernicious anemia
Cerebellar atrophy
IgA nephropathy, IgA glomerulonephritis
Myasthenia gravis
Partial lipodystrophy
Polymyositis
Primary biliary cirrhosis
Primary sclerosing cholangitis
Recurrent pericarditis
Relapsing polychondritis
Rheumatoid arthritis
Sjögren's syndrome
SLE
Type I (insulin-dependent) diabetes mellitus
Ulcerative colitis
Vasculitis (both systemic and cutaneous)
Vitiligo

Common genetic factors have been suggested as the reason for the frequent association of GSD with AI conditions (227), and the prevalence has been shown in some cases to be related to the duration of exposure to gluten (278). The range of disorders associated with TGc ELISA positivity may not be limited to those presented in this study. Indeed, sera from patients with linear IgA dermatosis, herpes gestationis, vasculitis other than Wegener's granulomatosis, IgA pemphigus, AI hepatitis, acute hepatitis A, and cholestatic hepatitis due to choledocholithiasis also were positive in the hTGc ELISA; however, these data are not presented because of the small number of patients. The frequent TGc ELISA positivity suggested either a general association of TGc with autoimmunity and cell destruction or an aspecific detection of IgA Abs in our assays.

While the elevation of total IgA Ab concentrations may result in a non-specific adsorption of IgA to the ELISA wells, this was minimized by saturating levels of the coating antigen and by dilution of the sera in solution containing Tween 20. One fifth of the tested AI sera had elevated total IgA levels, well under half the number showing raised concentrations of IgA Abs against TGc. Further, whereas both the AGA and the TGc ELISA positivity was often detected in connection with elevated total IgA concentrations, their correlation with IgA levels was weak (Fig. 20). In pilot studies, 0.2% BSA or 200 mM NaCl in TET was used for dilution of the sera and incubation in the ELISA wells. The albumin did not change the signals significantly. The NaCl reduced the reactivity of all sera with elevated IgA titres; however, although the degree of reduction depended on the IgA titre, it was independent of the diagnosis. Given these results, non-specific IgA adsorption is unlikely to be responsible for the positive results.

Reactivity with minor contaminants in the TGc preparations is more feasible because the gpTGc preparation was not purified to homogeneity, and although the hTGc preparation was purified so that a single band was seen on a Coomassie-stained gel (Fig. 15), this is not an evidence of perfect purity. The antigen in the endomysium has been reported to be identical to TGc (163, 162), but none of the false-positive sera showed endomysial staining.

There are studies of small numbers of cases where high TGc IgA ELISA titres occurred in non-GSD patients. In one study (71), EMA-negative patients had elevated IgA Abs in a gpTGc ELISA (number of patients in parentheses): SLE (3), primary biliary cirrhosis (1), Crohn disease (1). In a further study, two of eight patients with systemic AI diseases (SLE and Sjögren's syndrome) had IgA Abs against TGc (228). Five of 32 (15%) patients (four having Crohn disease and one ulcerative colitis) as well as 13 of 36 (36%) patients having chronic liver disease (alcohol cirrhosis, primary biliary cirrhosis, primary sclerosing cholangitis and AI hepatitis) were false-positive in a gpTGc ELISA (159). Recent studies described type I diabetes mellitus patients with elevated levels of IgA Abs against TGc who had normal duodenal architecture using traditional histological methods (159, 160, 177). Anti-TGc IgA has also been detected in two EMA-negative patients with non-Hodgkin's lymphoma (38). EMA-positive AI patients without GSD are only very rarely reported (37). Also serum IgG from one patient with Sjögren's syndrome, one with AI hepatitis, and five with Wegener's granulomatosis reacted with TGc (72).

In contrast, others failed to find increased anti-TGc IgA in children and young adults with type I diabetes mellitus (259) or in patients with ulcerative colitis and Crohn disease (10, 259, 270) in a gpTGc ELISA system.

In other studies, false positivity was detected only in the gpTGc ELISA suggesting that it is due to impurities present in the gpTGc preparation. High frequency of false-

positive results using a gpTGc ELISA was found in patients with elevated transaminases and chronic liver disease, and the presence of anti-gpTGc did not correlate with the presence of EMA positivity or histological evidence for CD (39). The false positivity disappeared, however, when hTGc was used. In a more recent study, 50% of EMA-negative patients with AI hepatitis or primary biliary cirrhosis and 6.5% with type I diabetes mellitus were found to be false-positive in a gpTGc ELISA whereas only 6% and none by a hTGc ELISA, respectively (53). In this study, no false-positive sera reacted with a protein band having the same migration speed on an immunoblot as TGc, but were immunoreactive with other bands. Some CD sera have previously been shown not to react with TGc in immunoblots, thus this method might not be appropriate for detection of anti-TGc reactivity (69, 72).

Given the high occurrence of TGc ELISA reactivity in AI diseases and that the EMA negativity cannot exclude the presence of low titre anti-TGc Abs together with additional Abs directed against minor contaminants, it is also possible that TGc might be a cofactor as well as a secondary autoantigen in a number of AI disorders. Wajda et al. suggested already in 1965 that TGc could play a role in AI diseases due to modification of proteins (281). Isoniazid and hydralazine, two drugs being relatively frequent inducers of SLE, can serve as amine substrates for TGc (187). MRL*Ipr/Ipr* mice, which have a defective CD95 receptor, suffer from an age-dependent, severe, SLE-like AI disease, and produce large amounts of autoantibodies against TGc (224). While these mice do not have any known defect in the TGc gene (60), TGc with no or very low activity accumulates abnormally in maturing lymphoid organs (224). The impairment of TGc-catalyzed polymerisation of proteins in apoptotic or necrotic cells leads to an increased liberation of both TGc and other cellular constituents (224), and this may induce an AI response. An AI response against TGc may aggravate insufficient TGc leading to a pathological cycle, thus Abs against TGc may facilitate or promote an AI disease, or their appearance during the course of an AI process may result in increased disease activity. Finally, TGc-/- mice develop anti-nuclear Abs after the first year of life, which might be the basis of an SLE-like disease of the mice (Fésüs L et al., personal communication).

In conclusion, the broad spectrum of diseases linked with TGc ELISA positivity suggests either a general association of TGc with autoimmunity and cell destruction or an aspecific detection of IgA Abs in our ELISAs. In order to clarify this situation, further experiments are planned in the near future.

6.4. Relevance of ELISA results

The results of our ELISAs were in good agreement with the histological diagnoses and the results of the EMA test confirming that TGc is a major autoantigen of GSD. The ELISA with the human antigen turned out to be slightly more sensitive in this regard than the EMA test or the gpTGc ELISA. Therefore, it is suitable for diagnosis, screening, and follow-up of GSD, although the AI study alerted us that false-positive results are possible in certain disease groups, the cause of which is currently unknown. The role of anti-TGc Abs in the etiology of GSD remains to be investigated.

We found in the gpTGc study that the median serum IgA Ab level against TGc was significantly higher in patients with untreated CD than with untreated DH, results in

agreement with those of Dieterich et al. (70), although this could not be confirmed in the hTGc study (a similar difference was found but it was not significant and the 95% CIs overlapped, see 5.2.2). This finding is supported by the observation that the EMA test is often false-negative in untreated DH patients.

Our gpTGc ELISA was able to show the effect of a GFD both in CD and DH patients early after the beginning of the GFD when the EMA test had not yet been turned negative (Fig. 14). This finding is important because the apparent advantage of the quantitative TGc ELISA against the semiquantitative EMA test is that the former allows the gastroenterologist to control the compliance to the GFD more effectively.

The 'Expression of the human tissue transglutaminase in human embryonic kidney cells and purification via StrepTactin affinity chromatography.' was patented by the European Patent Office (No. 99111975.1.). The human TGc ELISA presented in this work is currently produced on an industrial scale and commercially available.

7. CONCLUSIONS

TGc was proved to be the major autoantigen of GSD, although in DH, the dominant autoantigen responsible for skin symptoms was found to be a related isoenzyme, the TGe. It is now evident that serological tests based on indirect immunofluorescence on different tissues (umbilical cord, endomysium, jejunum, liver) detect TGc in these tissues, and the IgA Abs previously referred to as anti-endomysium, anti-reticulin or anti-jejunum Abs are identical. The observation that TGc is the major autoantigen of disease-specific Abs in GSD allowed the production of serological tests independent of animal tissues. In this work, the development of an ELISA based on the guinea pig and the human antigen has been presented and tested for usefulness in the clinical practice.

The results showed that both the gpTGc and the hTGc ELISA were specific and sensitive enough for use as a simple diagnostic, screening and follow-up method for GSD when compared to healthy controls. If compared to controls with non-AI diseases, the hTGc ELISA was found to be as specific and sensitive as the EMA test, and somewhat superior to the gpTGc ELISA. Our results showed the high diagnostic value of all tested systems in this study, in particular that of the hTGc ELISA, which had almost perfect sensitivity and specificity if compared with healthy controls, and did not have the practical disadvantages of EMA test. However, it is evident that in certain cases, in particular in active AI diseases, TGc ELISA positivity should not alone be taken as the basis for a diagnosis of GSD. Positive results in symptomless individuals should always be verified by EMA test before submitting them for jejunal biopsy, while negative results ought to be controlled if the clinical symptoms and signs are suspicious of GSD.

Thus one can conclude that the hTGc-based ELISA should be the method of choice for simple and non-invasive diagnosis, screening, and follow-up of GSD. The role of TGc in the etiology and pathogenesis of GSD (and possibly in other diseases) remains elusive, further studies are needed to understand whether IgA Abs against TGc have pathological consequences, prognostic uses or are only markers of gluten consumption.

8. ACKNOWLEDGEMENTS

First of all I would like to thank Prof. Dr. Sarolta Kárpáti for her inspiration. She was the person who impressed me with the importance of research on the role of transglutaminases in GSD. She provided me with the opportunity to use the patient sera she had collected, she was the person who organised contact to Prof. Paulsson and sent me to Cologne to spend one of the most useful and successful periods of my life. Her enthusiasm is fascinating, the energy she invests into research commands respect, and her indefatigable efforts to sow the seeds of high quality research into her Ph.D. students' heads is astonishing. Without her altruistic help and ambitious dreams, this project would not have begun.

I would like to acknowledge the continuous (both personal and financial) support of Prof. Attila Horváth. He has never let me feel he was being my employer, and offered me all the possibilities to develop my dermatology skills. His very (self-)critical and evidence based point of view in the dermatology helped me a lot in the realisation of facts among the impressions.

I am indebted to Prof. Mats Paulsson for his generous help throughout my two years' stay in Cologne, and for offering me the opportunity to perform these studies in his department. He guided my research in a very competent manner. The proficiency he managed to lead both scientifically, and financially, and bureaucratically the huge Institute for Biochemistry II, made him an outstanding example to the younger generation. I have never seen in my life such a person whose door, although being a director under extremely high work load, was practically always open for everyone. The eminently democratic atmosphere he provided ensured a uniquely free feeling of independence, and excellent environment in which to study. I learned that it was worth following his directions even if one was disagreed with him at the first time; being a real master of his profession, he had the special talent to intuitively know in which direction to go. I am also obliged for his prominent assistance to receive financial support especially at the beginning and in the second year of my stay. And last but not least, I will never forget that he was able to change my agitated, worrying style into a calmer and patient one, helped to clear my mind of unnecessary, complicated actions, and showed me the way of thinking of a generous, honest, flexible, and talented investigator.

I would like to express my gratefulness to Prof. Dr. Thomas Krieg for contacting Prof. Paulsson for the first time, for inviting me, after I had arrived, to participate on the visits and other activities in the Department of Dermatology, for his help receiving financial support, and for the critical discussions about our results, useful suggestions, and reviewing of our work.

I am especially grateful to Dr. Neil Smyth for acting as extremely competent scientific tutor and English teacher during the whole work. When I first met him, my written English was not bad, but it took months until I began to understand significantly more than 50% of what he told me. As a result of his effect, and like everyone in the lab in Cologne, today I not only understand, but also speak 'Neilglish', a Liverpool dialect with individual tone, intonation, accents and idioms, and I often realise that I do not know the Hungarian name of several objects in our lab in Budapest whereas I do know the English name for them. I usually regard people who declare they are always right as

conceited, but surprisingly enough, he was *really* always right when he pronounced he was sure! I also wish to thank for his investments in the numerous visits in pubs and restaurants of Cologne which always induced advantageous progress of research. I will never forget his words that everything should be done as quickly as possible which were not only a slogan but showed his uncommonly efficient way of working which impressed me. Special thanks for the CD rack, currently an important piece of furniture next to my computer desk, I received as a memory of someone using his talent not only in pipetting but also in carving ideas. And last but not least, I would like to express my gratitude for reviewing this manuscript.

I would like to acknowledge the help of Petronella Izbéki who not only provided optimal home background and atmosphere for effective work but also technical help organising the patients' data, the numerous copies of scientific papers, and slides. I am very grateful to my parents for supporting my studies, doing my business in Hungary when I was abroad, and helping me together with Petronella in the short period when I became disabled.

I thank Uwe Odenthal for his aid in learning the different techniques of molecular biology which was particularly important in the first few months, and for his generous gift, the expression vector pCEP-Pu/BM40SP/C-Strep, together with plenty of experience with using it. He always shared all of his materials with me. I will forever remember his kind personality. Being a genuine lad from 'Kölle', he helped every foreigner in adapting to the milieu of Cologne, he always found how to help, even without asking him (his aid in transporting furniture for me was vital), and provided a friendly and cheerful environment in the lab.

I am beholden to Barbara Merkl and Christian Frie for introducing me into the complicated world of a biochemical lab, especially into cell culture work, cloning, isotope techniques, and microscopy. Without their help I would have been lost there. How many time they spared me with knowing which reagent where can be found! I still try to eat vegetarian food as often as possible, and now I am deeply skilled in microphotography and the life of several insects.

I appreciate Getrud Pelzer's indispensable assistance in the bureaucratic labyrinth of the German rules. She was not only a skilful secretary but also a 'student-sitter' taking care for every important detail including a huge, delicate piece of ham at Christmas time.

I thank Nevres Ercan for her kind technical assistance and providing me with a lot of odds and ends around the flat.

I am very grateful to Drs. Judith-Antonia Lummerstorfer and Patrick Tunggal for their support sharing materials with me, showing tricky ways of doing experiments, providing me with chocolate and sweets, producing cheerful atmosphere in the workplace, and inviting me to play basketball together with the Tunggal family, Lutz and Lili, Thomas, Martin, Maria and many others which was one of my most important sport activities in Cologne. I am especially beholden to Lutz Reiche for his help after my disabling basketball accident. I am indebted to Dr. Budi Tunggal for his essential help in computer technical problems, and Dr. Lucy Tunggal for sharing reagents and incubation boxes with me.

I would like to acknowledge the help of Dr. Brigitte Ritter in many details of my work including the confocal microscopy, the site directed mutagenesis, and particularly her aid after midnight when usually nobody else than her was to be found in the institute. I will never forget the countless cinema visits and pizza-parties together with Uwe

– usually late in the night. I clearly remember her appreciative sense of humour that made every contact with her a pleasure.

I thank Dr. Jan Modregger for his help in fighting with hardware problems, for the useful instructions showing me how to use certain softwares, and for the generous gift of valuable reagents he had produced. I will never forget his unique sense of humour either which together with Brigitte's and Markus' allowed me to enjoy the atmosphere of every visit in Markus' lab. Special thanks for the photo album showing the life in the institute which I beleive contained mainly his photographs.

I thank Drs. Markus Plomann, Raimund Wagener, Robert Dinser, Harald Hambrock, Frank Zaucke, and Patrik Maurer for unselfish opening of the door to their wisdom.

I thank Ursula Canton for being my partner in the rock 'n' roll club and Karola Muth for teaching and training me there.

I am grateful to Dr. Márta Csikós for aid in performing ELISAs, the management of my official affairs in Hungary during my stay abroad, and the useful discussions. I would like to acknowledge Ferencné Menyhárt for preparing EMA tests and Prof. Dr. Sarolta Kárpáti and Dr. Klaudia Preisz for evaluating them. I thank Tamás Szaák for the DH patient's photos. I am indebted to Prof. Dr. Sarolta Kárpáti and Dr. István Kósnai for critically reviewing the manuscript of this thesis. I appreciate Dr. Krisztina Becker's efforts to solve bureaucratic problems in connection with the Ph.D. programme and the profitable discussions. I am thankful to all colleagues in our Hungarian 'IF' lab mentioned above and to all colleagues in our Department of Dermato-Venereology who enabled with their extra work that I could be absent for more than two years.

I thank Dr. Daniel Aeschlimann for the kind gift of full length cDNA of human TGc. I thank Dr. Marcus Macht for performing mass spectrometry; Drs. Tamás Zágoni, Christof Geisen, Erika Tomsits, Ulrich Töx, Nico Hunzelmann, and Prof. Jörgen Wieslander for providing patient sera. I acknowledge Elke Dietzel, Birgitta Jakob, and Charlotte Ziesemer for their technical assistance. I am grateful to Prof. Walter Lehmacher for statistical review of the manuscript and Dr. Martin Hellmich for his help in the statistical comparison of the two ELISA systems. I thank Drs. Ferenc Péterfy and Klára Rásky for their preliminary studies on the guinea pig TGc ELISA system.

I thank Prof. Dr. András Falus for his support to be admitted to the Ph.D. Doctoral School, for his help in the organisation of the final Ph.D. examination, and for regularly inviting me together with Marianna Holub to interesting scientific programmes, the 'Ph.D. Saturdays'.

I also have to acknowledge the support of several organisations. I was supported by a fellowship of the Deutscher Akademischer Austauschdienst (A/98/23048), the Deutsche Forschungsgemeinschaft (FOR 265), the Immundiagnostik AG, the European Science Foundation (Protein Cross-Linking – the Transglutaminases Programme), and the Maria Pesch Foundation. The study was supported by a common grant of the Deutsche Forschungsgemeinschaft and the Magyar Tudományos Akadémia (project 436 UNG 113/135/0, Pa 660/2-1), the Köln Fortune Program of the Medical Faculty of Cologne, the Friedrich and Maria Sophie Moritz Foundation, the Országos Mûszaki Fejlesztési Bizottság (OMFB 6197), and the University Scientific Grant (ETT 155/2000) of Semmelweis University.

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Publications in strong connection with this work are underlined, and a reprint is attached to the end of this document.

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