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RESEARCH ARTICLE

Celiac disease in patients with type 1 diabetes: a condition with distinct changes in intestinal immunity?

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Two common chronic childhood diseases—celiac disease (CD) and type 1 diabetes (T1D)—result from complex pathological mechanisms where genetic susceptibility, environmental exposure, alterations in intestinal permeability and immune responses play central roles. In this study, we investigated whether these characteristics were universal for CD independently of T1D association. For this purpose, we studied 36 children with normal small-bowel mucosa and 26 children with active CD, including 12 patients with T1D. In samples from the small-bowel mucosa, we detected the lowest expression of tight junction protein 1 (TJP1) mRNA in CD patients with T1D, indicating an increase in intestinal permeability. Furthermore, these samples displayed the highest expression of forkhead box P3 (FoxP3) mRNA, a marker for regulatory T cells, as compared with other patient groups. At the same time, serum levels of IgA antibodies specific for the CD-related antigens deamidated gliadin and tissue transglutaminase (tTG) were the highest in CD patients with T1D. In contrast, no significant differences were found in IgA or IgG antibodies specific for bovine beta-lactoglobulin or *Bifidobacterium adolescentis* DSM 20083-derived proteins. There were also no differences in the transamidating activity of serum autoantibodies between patients and control individuals. Our results show that patients with T1D and newly detected CD exhibit severely altered intestinal permeability, strong local immune activation and increased immunoregulatory mechanisms in the small bowel. Further study is required to determine whether these extreme changes in this CD subgroup are due to some specific environmental factors (virus infections), unknown genetic effects or autoimmune reactions to antigenic targets in intracellular tight junctions.
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Keywords: autoimmunity; celiac disease; immunoregulation; intestinal mucosa; type 1 diabetes

INTRODUCTION

With an approximate prevalence of 1.0% in Western countries, celiac disease (CD) is one of the most common lifelong chronic disorders.[1] CD and type 1 diabetes (T1D) form a significant sector of childhood diseases with high rate of co-occurrence.[2] Both diseases are autoimmune in origin and develop as a result of complex pathological mechanisms, involving several common genetic, environmental and immunological factors. Among these, the common susceptibility loci of HLA and other immune system-related genes,[3] permeability changes in the small-bowel mucosa,[4] and association with wheat consumption[4,5] are prominent. Both diseases are increasing in most regions of the world,[6,7] as are several other autoimmune diseases, but the actual cause of the disease remains unknown. However, several recent studies have highlighted the role of environmental and social changes that have taken place over the last several decades.[8,9]

The central event in the pathogenesis of CD is damage to the small intestinal mucosa that occurs after ingestion of gluten or related prolamins in genetically susceptible individuals. During this process, several morphological and immunological alterations have been demonstrated in the small-bowel mucosa. These mucosal changes are also reflected in the presence of characteristic circulating antibodies[10] directed against tissue (type 2) transglutaminase (tTG) and deamidated gliadin; these antibodies are specifically associated with small intestinal destruction in CD and are therefore widely used for serological CD screening.[11] Histologically, CD is characterized by intestinal villous atrophy with high numbers of infiltrating T cells of different lineages.[10,12] Recently, much interest has been paid to the characterization of $CD4^+$ T cells in the intestinal mucosa because the balance between inflammatory and regulatory cells is crucial in determining immune-mediated damage to the intestinal mucosal. Studies have demonstrated that the number of $CD4^+CD25^+FoxP3^+$ regulatory T cells is increased in the small-bowel mucosa of CD patients; this increase might represent a compensatory mechanism to downregulate the elevated immune response to foreign and self-antigens.[13–15]

Immune responses to the self-antigen tTG may be caused by the increased expression of tTG in the intestinal mucosa,[16] a conformational change in enzyme structure[17] or the release of intracellular tTG that, when picked up by dendritic cells, subsequently leads to the activation of adaptive immune responses. Antibodies against tTG may influence either the deamidating or the transamidating activity

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of the enzyme, which may lead to significant biological effects in intestine. However, results regarding the effect of tTG autoantibodies on enzyme function remain controversial.

Immune reactions directed against foreign antigens, such as food, might be at least partly dependent on the integrity of the intestinal epithelium.[18,19] Altered intestinal permeability in CD is well known, particularly in connection with gluten exposure that diminishes intestinal integrity while also activating localized immune responses.[20] Importantly, removal of gluten from the diet of CD patients normalizes intestinal permeability and prompts the return of normal structure within the intestinal mucosa; this parallels an increased expression of tight junction protein 1 (TJP1), also known as zona occludens 1, in the small bowel. However, electron microscopic analysis has shown that damage may be irreversible in a portion of patients.[21]

Similarly, intestinal mucosa from individuals at risk for T1D or with T1D exhibit abnormalities in intestinal permeability as evaluated by functional tests or electron microscopy studies.[22–24] In experimentally induced diabetes in rats or mice, changes in intestinal permeability are also detected before the onset of diabetes; increased intestinal permeability in these cases has been shown to be dependent on increased zonulin expression, which regulates tight junctions in the intestinal mucosa.[25–27]

Additionally, mucosal biopsy, fecal microbiota and serological studies have indicated that the composition of intestinal microbiota may influence the immune mechanisms that participate in the development of CD[28,29] and T1D.[27,29,30] Consistent with these studies, recent investigations have stressed the importance of *Bifidobacteria* species and strains in the modulation of immune reactivity at the intestinal mucosa level.[31] In particular, *Bifidobacterium adolescentis* has recently received attention because this bacterium is more prevalent in patients with allergic disorders compared to non-allergic subjects, thus indicating that it might be connected to the development of immune dysfunction.[32,33]

Based on available experimental and clinical results, it has been proposed that the pathogenesis of T1D is closely linked to events that take place in the intestinal mucosa, where complex interplay between the intestinal microbiota, gut permeability and mucosal immunity determines autoimmune damage to pancreatic beta cells.[34] In several associations, this has also been demonstrated in CD and other autoimmune diseases.[4,35]

Here, we aimed to assess whether differences in intestinal permeability, characterized by TJP1 mRNA expression, and intestinal regulatory T cells, measured by Foxp3 mRNA expression, as well as different serum antibodies levels exist between CD patients with and without accompanying T1D. Our task was also to evaluate differences in the effect of transamidating activity of serum tTG antibodies on tTG between patients with CD and controls.

MATERIALS AND METHODS

Samples

The study comprised three patient groups: (i) 12 patients with active CD and T1D (five males, aged 5–14 years); (ii) 14 patients with active CD and no T1D (aged 1–15 years, 9 males); and (iii) 36 patients with normal small-bowel mucosa revealed at biopsy for functional dyspepsia, ulcus duodeni, erosive gastritis, neurological disorders, allergic dermatitis, etc. (aged 1–18 years, 12 males). Patients with CD were identified from 291 childhood T1D patients (aged 2–18 years, 167 males) during retrospective and prospective studies assaying anti-tTG IgA and/or endomysium antibodies between 1995 and 2007 as described elsewhere.[36] Small-bowel biopsies (either with Watson capsule or gastroduodenoscope) were performed in Tartu University Children's Hospital for all patients. The Watson capsule biopsy samples were divided in two portions: one half was stored for morphological examination and the other half was immediately quick-frozen in TissueTek OCT Compound (Sakura, Finetek, Finland) and stored at −80 °C. At gastroduodenoscopy, one biopsy sample was used for morphological examination, while the other was stored in RNA*later* (Ambion Inc., Austin, TX, USA) at −25 °C for later analysis by RT-PCR. The small-bowel mucosa state was morphologically evaluated according to the Marsh classification,[37,38] and the diagnosis of CD was performed using the criteria of the European Society for Pediatric Gastroenterology, Hepatology and Nutrition.[39] None of the patients had received a diagnosis of CD before or had been on gluten-free diets. At the time of biopsy, all patients donated blood samples for antibody studies. This study was approved by the Ethics Committee for Medical Investigations at the University of Tartu. All studied children and their parents gave their written consent.

Detection of serum antibodies

IgA and IgG type antibodies against tTG were detected using ELISA and human recombinant tTG according to the method described by Teesalu *et al.*[40] To detect IgG anti-tTG antibodies, tTG was attached to microwells by human fibronectin (Sigma-Aldrich, St Louis, MO, USA).

IgA and IgG type antibodies to bovine beta-lactoglobulin (Sigma-Aldrich) were measured using an ELISA protocol suggested by Professor E. Savilahti from Helsinki University with minor modification. In brief, Nunc PolySorp microtiter plates (Roskilde, Denmark) were coated with bovine beta-lactoglobulin at a concentration of 1 µg/ml in phosphate-buffered saline (PBS) and incubated overnight at 4 °C. After being washed with PBS containing 0.1% Tween 20, plates were treated for 1 h at 37 °C with a blocking solution of 2% normal horse serum in PBS. Subsequently, serum samples diluted 1 : 20 in 1% horse serum PBS were incubated on the plate (two wells with the antigens and one without the antigen) for 1 h at 37 °C. After a washing, horseradish peroxidase conjugated rabbit anti-human IgG (DAKO, Glostrup, Denmark) or rabbit anti-human IgA (DAKO) was applied to the wells, followed by a 1-h incubation at 37 °C. After another washing, tetramethyl-benzidine liquid substrate (Sigma-Aldrich) was applied, and plates were incubated for 10 min. at room temperature. After reaction termination with 2 M H_2SO_4, the optical density of the reaction product was measured at 450 nm. The absorption value derived from the mean value of absorption in two wells with the antigen minus the absorption value in well without antigen was taken as the absorbance value of the studied sera representing the relative amount of antibodies in the studied sample.

IgA and IgG antibodies to deamidated gliadin were measured by ELISA using a Euroimmun GAF-3X Kit (Medizinische Labordiagnostika AG, Lübeck, Germany); the reactions were performed according to manufacturer's instructions. The results are expressed in RU/ml.

IgA and IgG antibodies against *Bifidobacterium adolescentis*-derived proteins were detected by immunoblot using bacterial cell lysates. Lysates were obtained by growing *B. adolescentis* (strain DSM 20083) in anaerobic conditions (5% CO_2, 5% H_2 and 90% N_2) on Wilkins–Chalgren medium (Oxoid Ltd, Hampshire, UK) for 48 h in an anaerobic glove box (Sheldon Manufacturing Inc., Cornelius, OR, USA). Cells were collected, suspended in PBS and washed three times.

Subsequently, cells were disrupted with 0.1 mm glass beads (Biospec Products, Bartlesville, OK, USA) in PBS in the presence of protease inhibitors (Complete Tablets; Boehringer Mannheim, Mannheim, Germany) on ice. Protein concentrations in the lysates were estimated by Protein Assay Solution (Bio-Rad, Hercules, CA, USA). Equal amounts of proteins from different preparations were mixed with 300 μl SDS-PAGE sample buffer (62.5 mM Tris (pH 6.8), 2.3% SDS, 5% 2-mercaptoethanol, 10% glycerine and a few grains of bromophenol blue) and heated for 15 min at 95 °C. Approximately 100 μg of total protein was loaded for each gel.

Bacterial cell lysate proteins were loaded on 5–20% gradient gels with a 5% concentrating gel with All Blue 10–250 kDa molecular weight (MW) markers (Bio-Rad) as standards; proteins were separated using the vertical electrophoresis system SE-600 (Hoefer, San Francisco, CA, USA) connected to a thermostat. The separated proteins were transferred to polyvinylidene difluoride membranes (0.45 μm pore size) using a semidry electroblotter (Hoefer) as described by Nilsson *et al.*[41] After transfer, the membranes were blocked twice. First, the membranes were incubated for 15 min in a solution containing 10 g/l polyvinyl pyrrolidone (MW: 40 kDa) and 25% methanol suspended in an ethanolamine/glycine buffer (pH 9.6) containing 12.2 g/l ethanolamine (Merck and Co., Whitehouse Station, NJ, USA) and 18 g/l glycine (Merck and Co.). Second, themembranes were incubated for another 15 min with the ethanolamine/glycine buffer containing Tween 20 (0.14%) and gelatin hydrolysate (5 g/l).[7,36]

After membranes were cut into 5 mm wide strips, the strips were incubated on a shaker overnight at 4 °C with sera or plasma and diluted 1 : 50 for IgA and 1 : 100 for IgG antibody assay in an incubating buffer composed of 1.25 g/l gelatin hydrolysate, 0.25 g/l Tween 20, 6.1 g/l NaCl (Merck and Co.) and 0.06 g/l Tris Base (Sigma-Aldrich). Strips were incubated with secondary anti-human IgA or anti-human IgG (diluted 1 : 500; DAKO) antibodies labeled with horseradish peroxidase for 1 h at room temperature; subsequent substrate reaction with carbazole in 50 nm sodium acetate buffer (pH 5.0) in the presence of hydrogen peroxide (0.04% 3-amino-9-ethylcarbazole and 0.015% hydrogen peroxide) was performed for 30 min. at room temperature. Extensive washes with PBS–Tween were applied between incubation steps. Reactions were terminated by washing the strips with distilled water.

Strips were scanned using a Bio-Rad GS-710 Imaging Densitometer (Bio-Rad). The relative MW of the bands was estimated with the Bio-Rad Quantity One Image Analysis software (Bio-Rad) according to the All Blue molecular markers.

Measurement of tTG antibodies effect on transamidating activity of tTG

Human recombinant tTG containing a C-terminal 6His-tag was expressed in *Escherichia coli XL1-Blue* and purified to over 90% homogeneity by two-step chromatography as described previously.[40] Briefly, 1 mg of tTG protein was coupled to 1 ml of NHS-activated Sepharose 4 Fast Flow Beads in 2 ml centrifuge columns (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Human serum (0.5 ml), diluted 1 : 4 in PBS, was passed through a 0.45 μm filter and applied to tTG-Sepharose column for 30 min. After column was washed four times with 3 ml PBS, bound antibodies were eluted with 2 ml of 0.1 M glycine, pH 2.5, and pH neutralized by adding 0.5 ml of 1 M Tris-HCl, pH 8.0. After dialysis against PBS using Pierce Slide-A-Lyzer Dialysis Cassettes (Thermo Fisher Scientific Inc., Rockford IL, USA), the final preparations of affinity purified antibodies were stored at −20 °C. Following each purification step, tTG-Sepharose columns were regenerated with 6 M guanidinium chloride. Protein concentrations in antibody samples were determined by the Bradford method using bovine γ-globulin as a standard.

Tissue transglutaminase transamidation assays were performed using fibronectin bound tTG as described earlier by Király *et al.*[42] with minor modifications. Briefly, universal binding 96-well microtiter plate (Thermo Fisher Scientific Oy, Vantaa, Finland) wells were coated with 100 μl human fibronectin (Sigma-Aldrich) at a concentration of 5 μg/ml in carbonate/bicarbonate buffer (pH 9.6) overnight at 4 °C. The wells were washed five times with 300 μl TBS-T buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4) and human recombinant tTG (Teesalu, 2009) was added at 1 μg/ml in TBS with 2 mM DTT for 30 min at 20 °C. After being washed with TBS-T, the wells were incubated with 10 μg/ml of human anti-tTG, rabbit polyclonal anti-tTG (Thermo Fisher Scientific, Fremont, CA, USA) or CUB7402 monoclonal antibodies (5 μg/ml) in TBS, with 1% BSA and 2 mM DTT for 30 min at 20 °C. Next, after washing with TBS-T, 100 μl of 0.5 mM 5-(biotinamido)-pentylamine (Pierce Biotechnology) in reaction buffer (100 mM Tris-HCl, pH 8.5, 5 mM $CaCl_2$, 10 mM DTT) was added and plates were incubated for 15 min at 37 °C. The reactions were stopped by washing the wells with TBS-T five times, followed by incubation with streptavidin-conjugated alkaline phosphatase (Pierce Biotechnology) at dilution 1 : 2000 for 30 min. The absorbance was read at 405 nm with 492 nm subtraction. The binding of tTG is expressed as percentages of results without antibodies in inhibition experiments. The results are expressed as percentages of OD values obtained at the same conditions but without the addition of antibodies or inhibitors.

Detection of FoxP3 and TJP1 mRNA expression in small-bowel mucosa samples

Total RNA was isolated from biopsy samples using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. From successful isolations, cDNA was prepared using the cDNA Transcription Kit (Applied Biosystems, Foster City, CA, USA.) according to the manufacturer's protocol. Genomic DNA was eliminated by DNAase I treatment (Roche Diagnostics, Mannheim, Germany) prior to cDNA synthesis. Random hexamers (Applied Biosystems) were used to prime first-strand synthesis, and the reaction was performed in a total volume of 20 μl with the Multiscribe Reverse Transcriptase enzyme according to the manufacturer's protocol (Applied Biosystems).

Transcription level of the *FoxP3* gene (Hs00203958_m1), as a characteristic of regulatory T cells, was evaluated using TaqMan Gene Expression assay (Applied Biosystems) as described previously.[14]

The transcription level of the *TJP1* gene (Hs.510833) was evaluated using SYBR Green RT-PCR Gene Expression Analysis (SuperArray; Bioscience Corporation, Frederick, MD, USA). A total of 1 μl of cDNA from each sample was used for each duplicate measurement for human TJP1 (Hs.510833, SuperArray) in combination with 1 μl cDNA to measure the housekeeping gene β-actin (ACTB, Hs. 520640). RT-PCR gene expression analysis was performed according to the manufacturer's instructions using SYBR Green/ROX qPCR Master Mix (SuperArray; Bioscience Corporation). The homemade exogenous cDNA calibrator sample of TJP1 (prepared from Caco-2 cells) was used for the interassay standard to which all other normalized samples were compared. The Caco-2 cell line is known to express high level of TJP1 mRNA.[43] Total RNA was isolated from Caco-2 cells

using the RNeasy Mini Kit (QIAGEN GmbH) and QIAshredder Spin Columns (QIAGEN GmbH). The TJP1 cDNA was prepared from Caco-2 cells using the cDNA Transcription Kit (Applied Biosystems) according to the manufacturer's protocol.

The ABI Prism 7000 Sequence Detection System was programmed for 10 min at 95 °C, followed by 40 thermal cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification was performed in a total volume of 25 µl. Each measurement was performed in duplicate.

In both assays, for either *FoxP3* or *TJP1*, the comparative threshold (C_t) method was used to quantitate gene transcription in the samples. The C_t of controls (18S RNA for FoxP3 and Caco-2 cells RNA for TJP1) was subtracted from the target gene C_t. The obtained difference was the ΔC_t value. The ΔC_t of the calibrator was then subtracted from the ΔC_t of the sample. The obtained difference was called the $\Delta\Delta C_t$ value, and the results were expressed in relative units based on the calculation of $2^{-\Delta\Delta Ct}$, which yielded the relative amount of the target gene normalized to the corresponding control and relative to the calibrator. For the graphical FoxP3 representation, the relative numbers were multiplied by 1000.

Transcription levels of the *FoxP3* gene in the biopsy samples positively correlate to the number of $CD4^+CD25^+FoxP3^+$ regulatory T cells stained in the respective mucosal specimens,[14] whereas according to our experience with TJP1 immunocytochemical staining, differences between individuals with different *TJP1* gene transcription levels in the intestinal mucosa are rather complicated to evaluate due to variable staining patterns on the same sample.

Statistical analysis

The results obtained for different study groups were presented as mean±s.e. and compared using the *t*-test and the analysis of variance by ANOVA as well as by the two-tailed Spearman's rank correlation test. The age adjustment for mean values of antibody level was performed using multiple regression analysis. Differences were considered statistically significant at $P<0.05$. Statistica 8.0 software was used for all analyses.

RESULTS

Comparative expression of TJP1 and FoxP3 mRNA in the small-bowel mucosa and tTG

The lowest values of TJP1 mRNA expression were found in patients with CD and T1D (437.9±52.5); this group also exhibited the highest expression of FoxP3 mRNA (335.3±102.6). In contrast, the small-bowel mucosa of control individuals exhibited the highest TJP1 mRNA expression (713.2±98.4) and the lowest FoxP3 mRNA expression (61.1±10.1) (Figure 1). In patients with CD without T1D, the mRNA expression profiles of TJP1 and FoxP3 were between the two extremes, CD patients with T1D and control individuals. Overall, a significant negative correlation was found between TJP-1 and FoxP3 mRNA expressions in the biopsy specimens when all results were analyzed ($r=-0.42$, $P=0.003$).

IgA and IgG type antibodies against deamidated gliadin, tTG and beta-lactoglobulin

Serum levels of IgA and IgG antibodies against deamidated gliadin were significantly higher in CD patients and CD patients with T1D compared to patients with a normal small-bowel mucosa (Figure 2). In patients with CD and T1D, the levels of IgA antibodies against deamidated gliadin were significantly higher compared with CD patients without T1D ($P=0.003$). Using multiple regression analysis, age-adjustment showed that the values of deamidated gliadin-specific IgA and IgG antibodies were not dependent on age ($\beta=0.15$, $P=0.35$ and $\beta=0.048$, $P=0.76$, respectively). However, there was a significant negative correlation between the expression of TJP1 mRNA and

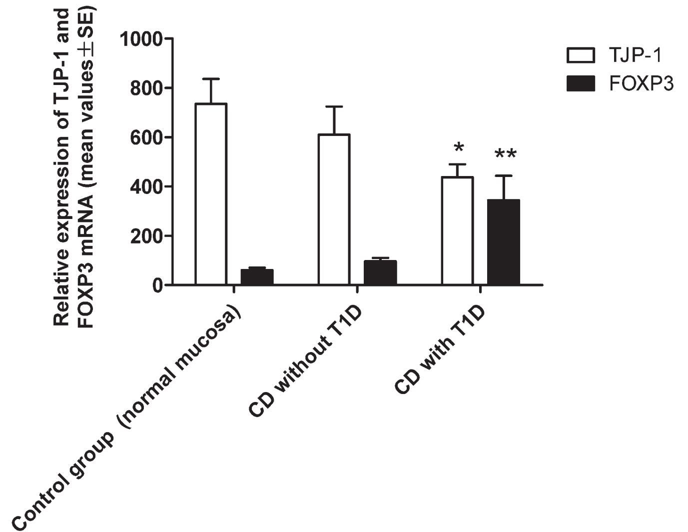

Figure 1 Relative expression of TJP1 mRNA and FoxP3 mRNA in small-bowel biopsy samples in children with normal intestinal mucosa and children with subtotal villous atrophy with or without T1D. *$P=0.01$, **$P=0.0001$, for comparisons between the control group (normal intestinal mucosa) and children with T1D. CD, celiac disease; FoxP3, forkhead box P3; T1D, type 1 diabetes; TJP1, tight junction protein 1.

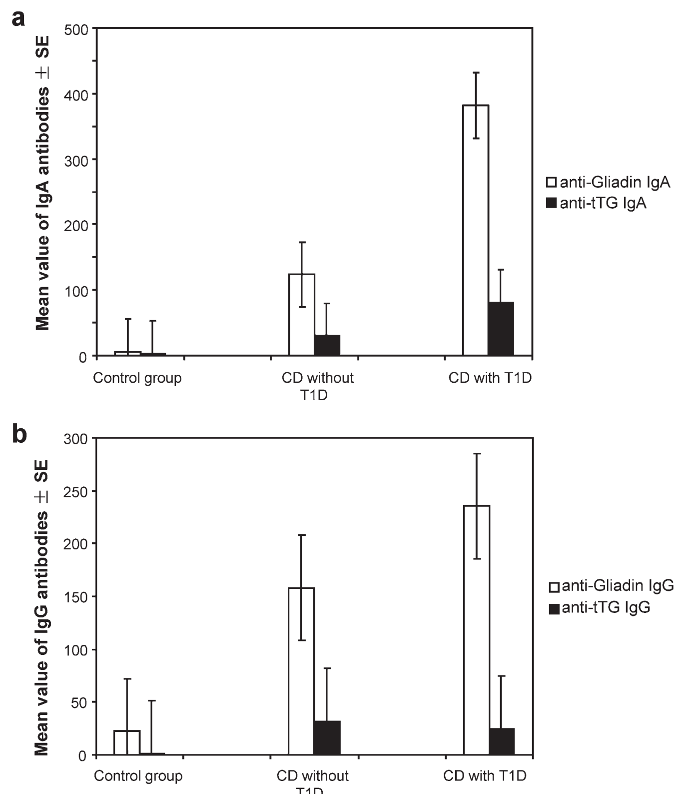

Figure 2 Comparative values (mean±s.e.) of IgA (**a**) and IgG (**b**) antibodies against deamidated gliadin and tTG expressed in RU/ml and AU, respectively. CD, celiac disease; tTG, tissue transglutaminase.

the level of deamidated gliadin-specific IgA antibodies ($r=-0.24$, $P=0.03$). The highest level of tTG-specific IgA antibodies was detected in CD patients with T1D (81.2±13.9). Patients with CD alone had significantly lower IgA type tTG antibody levels (30.0±12.9; $P=0.01$). Levels of IgG antibodies against tTG did not differ between CD patients with and without T1D. In CD patients, the levels of anti-tTG IgA antibodies were inversely correlated to TJP1 mRNA expression in biopsy specimens ($r=-0.84$, $P=0.03$); this correlation is also independent of patient age ($\beta=0.06$, $P=0.7$). The level of IgA, but not IgG antibodies against bovine beta-lactoglobulin, was significantly elevated in CD patients compared with patients with small bowel normal mucosa ($P=0.01$ and $P=0.21$, respectively; Figure 3).

Influence of tTG antibodies on the transamidating activity of tTG
The effect of anti-tTG antibodies on the transamidating activity of tTG did not differ between CD patients with and without T1D. In both groups, the effect of tTG specific antibodies on the transamidating activity of tTG was found to be not significant and within the range of the effect demonstrated by normal sera (86–98%).

Antibodies reacting with B. adolescentis-derived proteins
Altogether, a total of 43 different proteins with molecular masses ranging between 13 and 90 kDa from *B. adolescentis* lysate reacted with serum samples in the immunoblot assay. The reactivity pattern among different individuals within separate groups was very diverse. Overall, serum IgG antibodies were more reactive than IgA antibodies. *B. adolescentis* proteins with molecular masses between 29 and 42 kDa were more reactive as compared with other proteins. However, no differences in reactivity toward individual proteins between the groups or the immunoglobulin isotypes were revealed. Similarly, the mean number of reactive proteins between groups of subjects with normal small-bowel mucosa (control group) and those with subtotal villous atrophy was not statistically different. However, the mean number of reactive bands was somewhat higher in patients with abnormal intestinal mucosa as compared with normal individuals; for example, IgA antibodies revealed 7.1±3.3 versus 5.5±2.4 reactive bands, while IgG antibodies revealed 12.4±4.0 versus 8.7±6.1 bands, respectively.

DISCUSSION

As an autoimmune disorder, CD tends to be associated with other diseases that are autoimmune in origin. Among these, T1D is set apart because the prevalence of CD among T1D patients has been reported

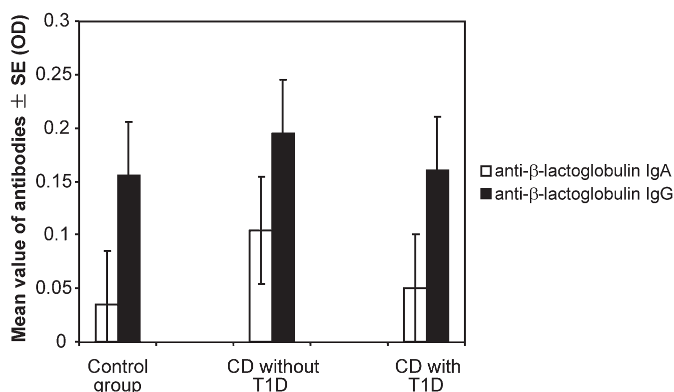

Figure 3 Comparative values (mean±s.e.) of anti-beta-lactoglobulin IgA and IgG antibodies expressed in OD units. CD, celiac disease.

to be about 10 times higher compared to that in the general population.[44] Therefore, it is plausible to hypothesize that this frequent association is due to common determinants that operate in both diseases. Indeed, there are at least seven common genetic loci determining the susceptibility of both diseases.[3] However, this finding cannot explain several characteristics of the association between CD and T1D, for example, why T1D patients are more prone to develop CD than CD patients to develop T1D.[2] It is also unclear why patients with recently developed T1D have a higher likelihood of developing CD than patients with long-lasting T1D.[36,45] It is possible that a subgroup of T1D patients might have certain specific characteristics that condition the development T1D and CD in the same person.

As the intestinal mucosa appears to play a central role in the pathogenesis of both T1D and CD, we investigated individuals with normal small-bowel mucosa and patients with intestinal villous atrophy representing CD with or without T1D. Comparing the expression of TJP1 mRNA as a marker of intestinal mucosa integrity, we revealed that intestinal permeability was likely to be most severely impaired in patients with T1D and CD. As a possible reaction to these mucosal changes and the related increase of antigenic pressure through impaired intestinal mucosa barrier function, the FoxP3 mRNA expression as a reflection of number of $FoxP3^+$ regulatory T lymphocytes was the highest among patients with T1D and CD. Also, levels of IgA antibodies directed against the CD-specific antigens tTG and deamidated gliadin were the highest in CD patients with T1D. These findings support the hypothesis that the immune system in the intestinal mucosa in such patients is highly activated due to the disturbed integrity of intestinal barrier function. Whether this is due to specific environmental or genetic factors or autoimmune mechanisms directed toward central molecules in the function of the intestinal mucosa (possibly associated with tight junctions) is not known.

First, a portion of T1D patients have genetically or environmentally conditioned high expression of zonulin, a molecule that has rapid and reversible effects on intestinal tight junctions' TJP1.[4] Indeed, studies on the development of diabetes in BBreeding (BB) rat and humans have revealed that impairment of intestinal integrity is zonulin-dependent. Furthermore, zonulin is liberated in high amounts during infections, which fits well the theory about the involvement of intestinal infective agents in the development of T1D.[4,26,46] However, we did not have the ability to study zonulin levels in the present study. It would be of particular interest for further studies to determine whether CD patients with or without T1D have different serum zonulin levels and whether these levels are related to the presence of infectious agents (rotaviruses, enteroviruses, etc.) in the intestinal mucosa of studied CD patients.

Second, one could propose that there were significant differences in microflora composition between CD patients with and without T1D despite our present finding that there were no statistical differences between controls and patients. In our study, we examined only one commensal strain of *Bifidobacterium* that does not reflect the full scenario of changes in intestinal mucosa. However, the *B. adolescentis* strain utilized has been convincingly demonstrated to be connected with the development of immune dysregulation in immune-mediated diseases.[32,33] However, it has also been shown that the ability of *B. adolescentis* derived proteins to stimulate the immune system is strain specific and geographically variable.[30] The great variation in the levels of IgA and IgG antibodies directed against the *Bifidobacteria* strain between individual patients indicates a dependence of the immune response on host-specific commensal microbiota. Furthermore, the results of somewhat higher levels of IgA antibody reactivity among patients with CD and T1D compared to controls support the rationale

to continue such studies in a larger context using other *Bifidobacteria* spp. in parallel. In recent T1D and CD investigations, intestinal microbiota have received attention as possible modulators of disease development.[27–30] However, one could ask whether the detection of serum antibodies against antigens of a particular commensal bacteria reflects its presence and involvement in immune reactions at the intestinal level. Although it is possible that we detected antibodies induced by antigens that originated from other microorganisms, we are still confident that a portion of the detected antibodies was directed against *B. adolescentis* itself or other *Bifidobacterium* spp. The role of different strains of *Bifidobacteria* in modulating gliadin induced inflammatory responses in intestinal epithelial cells has been recently demonstrated *in vitro*.[47] For further studies, more information about the specific proteins of *B. adolescentis* will be required.

Third, it is possible that autoantibodies against tTG have a different role in CD alone compared to CD developed in patients with T1D. Our study results, however, did not reveal significant differences between sera obtained from controls and CD patients with and without T1D as evaluated by their tTG transamidating capacity in vitro. At the same time we do not know whether there were differences between control and patient serum samples in the deamidating capacity of tTG.

Still, one cannot exclude the presence of specific autoimmune reactions in patients with CD and T1D association. T1D patients have antibodies against different wheat proteins in the absence of CD.[48,49] Although it could be taken as a secondary phenomenon related to increased intestinal permeability, recent studies concerning immunological crossreactivity between wheat proteins and self-proteins in T1D[50] suggest that autoimmune reactions to unknown antigenic proteins in intestinal mucosa might also be involved in small-bowel mucosa destruction in some T1D patients.

Although autoantibodies against the intestinal permeability modulating molecule zonulin have also been detected in patients with CD and T1D,[51] the actual role of these autoantibodies in the development of these diseases remains to be investigated.

Taken together, our investigation demonstrates that in patients with T1D, the development of CD is combined with changes in the small-intestine mucosa characterized by increased intestinal permeability, activation of the humoral immune system with elevated levels of IgA and increased mRNA expression of the regulatory cell marker FoxP3. Further study is required to determine whether these extreme changes in this CD subgroup are due to specific environmental factors, intestinal microbiota, unknown genetic effects or autoimmune reactions to novel antigenic targets in intercellular tight junctions or related structures.

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