“FEDERICO II”

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PhD Program
“Human Reproduction, Development and Growth”

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PhD Thesis

Celiac disease and Food Allergy: roles of undigested food peptides

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CHAPTER I

Celiac Disease

Coeliac disease (CD) is a systemic immune-mediated disorder elicited by gluten in genetically susceptible individuals. All patients with CD have a variable combination of gluten-dependent clinical manifestations, specific autoantibodies (anti-tissue transglutaminase/anti-endomysium), HLA-DQ2 and/or DQ8 haplotypes and different degrees of enteropathy, including severe villous atrophy, intraepithelial lymphocytes infiltration and crypt hyperplasia. These features are used to classify CD using the Modified Marsh Classification (Oberhuber) (1).

CD is considered a disease that involves both acquired and innate cell mediated immunity (2).

The gluten peptides, after crossing the epithelium into the lamina propria, are deamidated by tissue transglutaminase and then presented by DQ2+ or DQ8+ antigen-presenting cells to pathogenic CD4+ T cells (3,4). Once activated, the CD4+ T cells drive a T-helper-cell type 1 response that leads to the development of coeliac lesions. Activated gluten-reactive CD4+ T-cells produce high levels of pro-inflammatory cytokines, thus inducing a T-helper-cell–type-(Th)1 pattern dominated by interferon gamma (IFN-γ). Th-1 cytokines promote inflammatory effects including fibroblast or lamina propria mononuclear cell (LPMC) secretion of matrix metalloproteinases (MMPs), which are responsible for degradation of extracellular matrix and basement membrane, and increased cytotoxicity of intraepithelial lymphocytes (IELs) or natural killer (NK) T cells. These latter facilitate the apoptotic death of enterocytes by the Fas/Fas ligand (FasL) system, or interleukin 15 (IL-15)(5). IL-15, produced mainly by monocytes/macrophages, dendritic and epithelial cells, is a pro-inflammatory cytokine involved both in adaptive and in innate immunity. Memory

CD4+ and CD8+ T cells, NKT, NK, and TCRγ/δ+ cells expand following stimulation with IL-15 (6). Furthermore, IL-15 is also a potent growth factor for the intraepithelial lymphocytes (IEL) (7), whose density is significantly increased in CD, representing its hallmark. IL-15 is highly expressed not only in lamina propria cells but also in the intestinal epithelium of untreated CD patients and in patients with refractory celiac sprue, a premalignant condition, characterized by a massive IEL infiltration in particular of abnormal TCRγ/δ+ cells (8). The finding that in CD IL-15 is not secreted but bound to enterocyte cellular membrane, strengthened the hypothesis that IL-15 produced by epithelial cells, could be the main factor orchestrating the selective expansion of IEL, particularly TCRγ/δ+ and CD8 + TCR α/β+ lymphocytes bearing the CD94 NK receptor (9,10). It is noteworthy to mention that, in normal condition, intestinal TCRγ/δ+ cells recognize stress-inducible, MHC Class I-like molecules MICA and MICB, expressed on damaged epithelial cells (11). MICA and MICB proteins interacting with NKG2D receptor expressed on TCRγ/δ+ and NK cells, are found to activate innate cytotoxic and cytokine production responses. By contrast, the MICA ligation of NKG2D on CD8 + TCR α/β+ enhances the adaptive, antigen-specific cell-mediated responses (12). Importantly, a very recent study indicate that MICA molecules are strongly expressed on epithelial cells of CD patients with acute disease and upregulated both in the epithelium and LP of treated following gliadin peptide challenge (13). Also in this case IL-15 seems to play a crucial role. These studies pinpoint the fundamental role of innate immune response in the damage of intestinal mucosal tissues in CD, primarily due to the cytolysis of epithelial layer mediated by MICA/NKG2D activated IEL (13)

Interferon alfa (IFN-α) released by activated dendritic cells perpetuates the inflammatory reaction by inducing CD4+ T cells to produce IFN-γ. Additionally, through the production of Th-2 cytokines, activated CD4+ T-cells drive the activation and clonal expansion of B

cells, which differentiate into plasma cells and produce antigliadin and anti-tTG antibodies(14). By interacting with the extracellular membrane-bound tTG (mtTG), tTG-autoantibody deposits in the basement-membrane region might induce enterocyte cytoskeleton changes with actin redistribution and consequent epithelial damage (5).

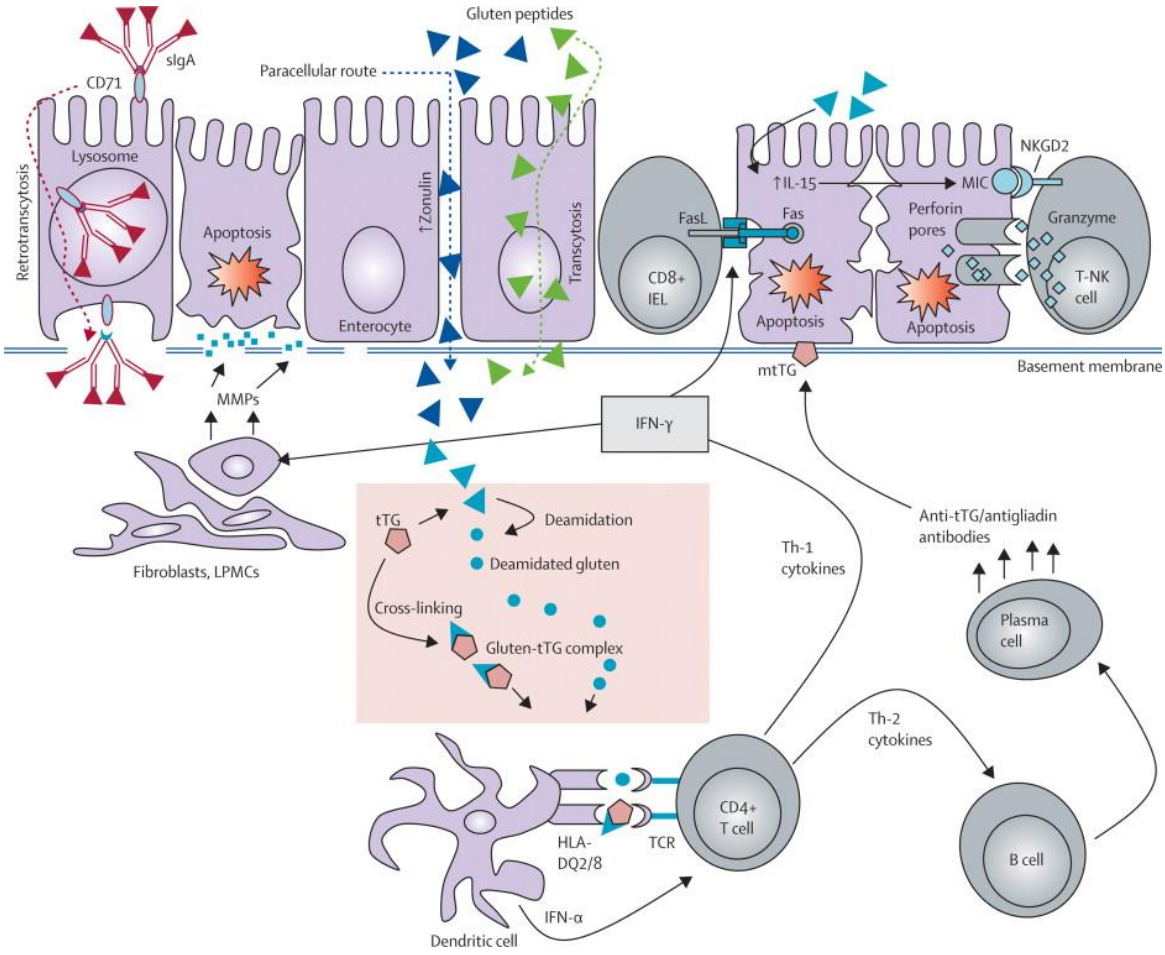


Figure 1. Mechanisms of mucosal damage in coeliac disease

1. The role of undigested gluten peptides

The term gluten mainly refers to wheat storage proteins. It is an extremely heterogeneous mixture of proteins, which are classified as gliadins and glutenins. Gliadins are monomeric proteins that are subdivided into α/β-, γ-, and ω-fractions according to their electrophoretic profile. Glutenins are present as polymeric complexes that are comprised of two subunit types, the high molecular weight and the low molecular weight subunits (15-16). A common feature of gluten proteins is the presence of a high level of proline and glutamine residues, which renders gluten highly resistant to proteolytic degradation in the gastrointestinal tract.(17,18). Consequently, the released peptides can cross the gut epithelial barrier and reach the lamina propria of the intestinal mucosa, triggering two immunological pathways: the adaptive and the innate immune responses (19).

Tissue transglutaminase (TG2), a Ca++ dependent enzyme, catalyzes both in vivo (epithelial brush border and subepithelial TG2) and in vitro the de-amidation of specific glutamine residues to glutamic acid, and enhances stimulatory capacity of gliadin-derived peptides by strengthening the binding to the HLA-DQ2/8 grooves (20). Recent studies have identified in the sequence motifs QXP the glutamine residues which are preferentially substrate of TG2-mediated deamidation. This represents an important tool for the prediction of toxic gliadin peptides (21). Although there are at least 50 T cell stimulatory epitopes in gluten proteins, a unique 33-mer peptide is the more immunogenic since it harbors six in part overlapping epitopes; moreover, it is resistant to the enzymatic degradation by gastric, pancreatic and brush border peptidase (22). It might reach in an intact and stimulatory form the immune districts of intestinal mucosa (22,23); furthermore, the 33-mer peptide does not require further processing in APC for T cell stimulation as it binds to DQ2 molecules with a pH profile that promotes extracellular binding (24).

The A-gliadin 33-mer have been reported to stimulate CD4+ T lymphocytes selectively isolated from small intestinal mucosa of CD patients (22,25,26,27). Importantly, T cell lines and clones from intestinal mucosa of CD patients recognize gliadin-derived peptides in the context of the disease-associated HLA-DQ2 and -DQ8 restriction molecules. By contrast, no evidence of T-mediated reactivity against dietary gliadin has been reported in normal, non-celiac mucosa (28,29). Moreover, it has been shown that gliadin-specific T lymphocytes from CD intestinal mucosa are mainly of Th1/Th0 phenotype and release, following gliadin recognition, prevalently pro-inflammatory cytokines, dominated by γ-interferon (28,30,31). γ-Interferon-dependent signaling pathways have been found to be enhanced in CD. Signal transducer and activator of transcription 1 (STAT1) (32) and interferon regulating factor 1 (IRF1) have both been found more expressed in untreated CD and in treated CD mucosa in vitro challenged with gliadin (33).

CD8+ T lymphocytes recognize peptides of 8–12 aminoacid length in the context of HLA Class-I molecules. Peptides interact with HLA-Class-I molecules through specific aminoacid and in determined positions known as binding motifs which vary depending on the type of HLA Class-I molecules (34). On the basis of the capacity of a panel of gliadin peptides to bind HLA-A2.1 molecules, we recently identified a peptide, mapping the 123–132 position of A-gliadin (A-gliadin 123–132, pA2) which is selectively recognized by CD8+ T lymphocytes from HLA-A2.1-positive celiac patients (35). This peptide induced γ-interferon production and cytotoxic activity by peripheral blood mononuclear cells from treated CD patients.

More recently, attention has been directed to the possible involvement of innate immune mechanisms in CD (36,37).

In particular, Maiuri et al. (37) showed that the synthetic peptide α-gliadin 31–43 (LGQQQPFPPQQPY) is able to upregulate the expressions of interleukin (IL)-15, of the

enzyme cyclooxygenase-2 (COX-2) and of the cell activation markers CD25 and CD83 on LP macrophages, monocytes, and dentritic cells, without stimulating CD4+ T-cells.

Barone et al. investigated the early events of celiac disease and in particular the interaction between gliadin peptides and intestinal epithelial cells. They found that the gliadin toxic peptide (P31-43) delays endocytic vesicle maturation and consequently reduces epidermal growth factor receptor (EGFR) degradation and prolongs EGFR activation, which in turn results in increased cell proliferation and actin modifications in celiac crypt enterocytes and in various cells lines (38). P31-43 enters CaCo-2 cells and intestinal enterocytes, interacts with early endocytic vesicles (39,40), reduces their motility and delays their maturation to late endosomes (39). Taken together, this information points toward an effect of certain gliadin peptides, i.e., P31-43, on endocytic function and indicates epidermal growth factor (EGF) signalling as one of the major pathways in the celiac intestine.

In the 2011 Barone et al. (41) showed that P31-43 induces at least two main effects by altering the trafficking of cell vesicular compartments. This leads to overexpression of the trans-presented IL-15/IL5R alpha complex, an activator of innate immunity, and, due to cooperation of IL-15 and EGFR, the proliferation of crypt enterocytes with consequent remodelling of the CD mucosa.

1.1 Alanine-scanning P31-43

Alanine-scanning mutagenesis is a simple and widely used technique in the determination of the catalytic or functional role of protein residues. Alanine is the substitution residue of choice since it eliminates the side chain beyond the β carbon and yet does not alter the main-chain conformation (as can glycine or proline) nor does it impose extreme electrostatic or steric effects.

In order to gain insight on the structural details that make P31-43 able to activate innate immunity mechanisms, in this study we analyzed and compared the structural and biological properties of P31-43 peptide with those of its Ala-scanning mutants(Figure 2). We focused on identification of the critical amino acid residues of P31-43 that have a toxic functions.

P3143 LGQQQPFPPQQPY

Ala1 AGQQQPFPPQQPY
Ala2 LAQQQPFPPQQPY
Ala3 LGAQQPFPPQQPY
Ala4 LGQAQPFPPQQPY
Ala5 LGQQAPFPPQQPY
Ala6 LGQQQAFPPQQPY
Ala7 LGQQQPAPPQQPY
Ala8 LGQQQPFAPQQPY
Ala9 LGQQQPFPAQQPY
Ala10 LGQQQPFPPAQPY
Ala11 LGQQQPFPPQAPY
Ala12 LGQQQPFPPQQAY
Ala13 LGQQQPFPPQQPA

Figure 2. Alanine scanning mutants of P31-43

The conformational behaviors of the peptides were defined by considering both chromatographic and NMR spectroscopic data.

HPLC profiles of P31-43 and almost all its Ala-mutants show two partially resolved peaks (Figure 3). We assumed that this atypical behavior was most likely a consequence of isomerization of two conformers, which is the result of the slow cis-trans isomerization of one of the four proline residues occurring in the sequences. For different peptides, it has been demonstrated that two adjacent prolines may cause the presence of slow interconverting cis–trans isomers with respect to the amide bond in solution.(42,43). The partial rotational hindrance around the Pro–Pro bond may explain the slow mutual conformational interconversion that causes peak splitting during HPLC analysis. Mutation of each residue in the synthetic peptide resulted in a unique single peak (Figure 3). Based on the chromatographic behaviors of the peptide analogues, it was deduced that the Xaa_Pro bond with exceptionally low rate of isomerization (ms) is contained in the trait FP8P9, and particularly involves P8.

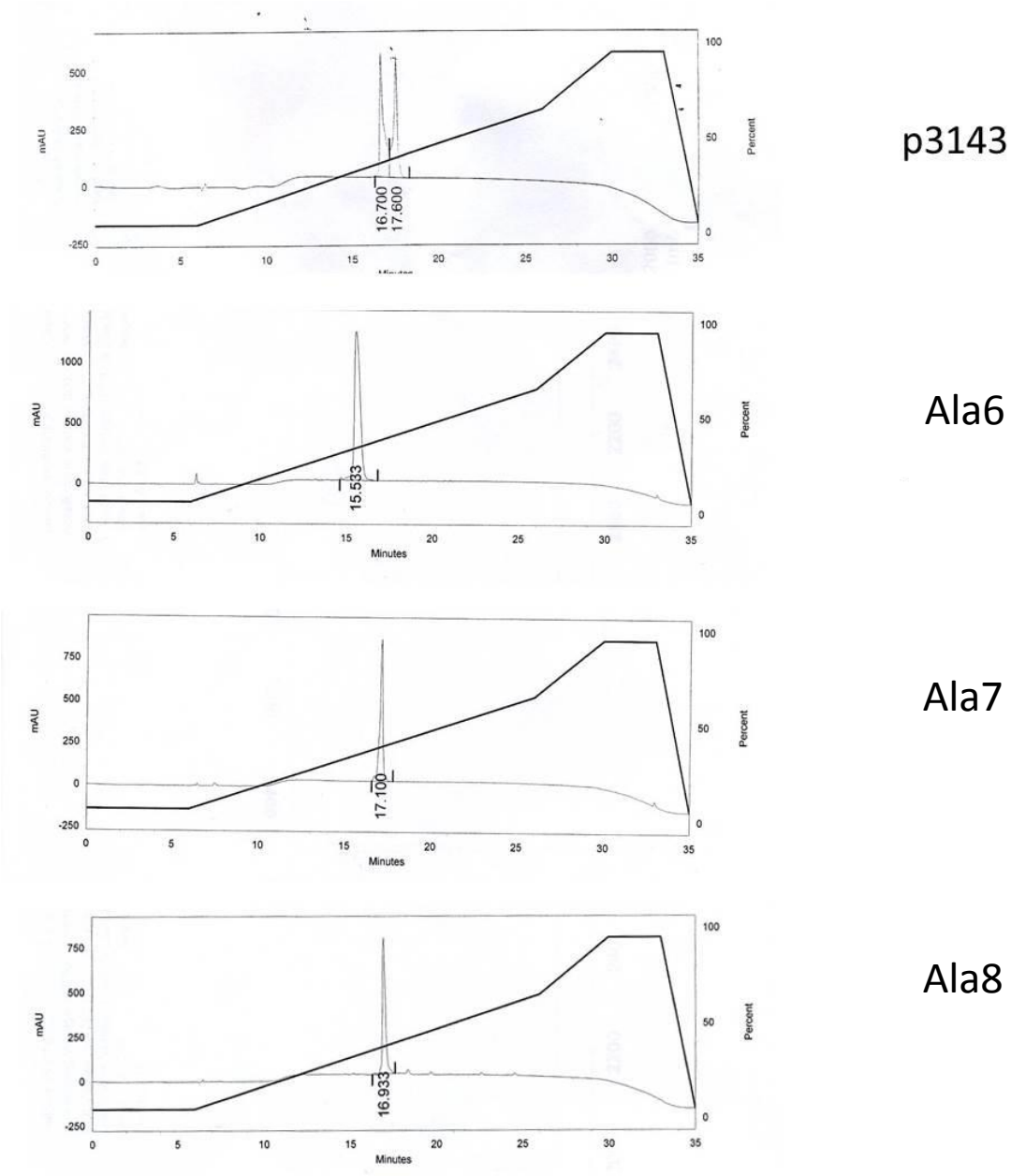
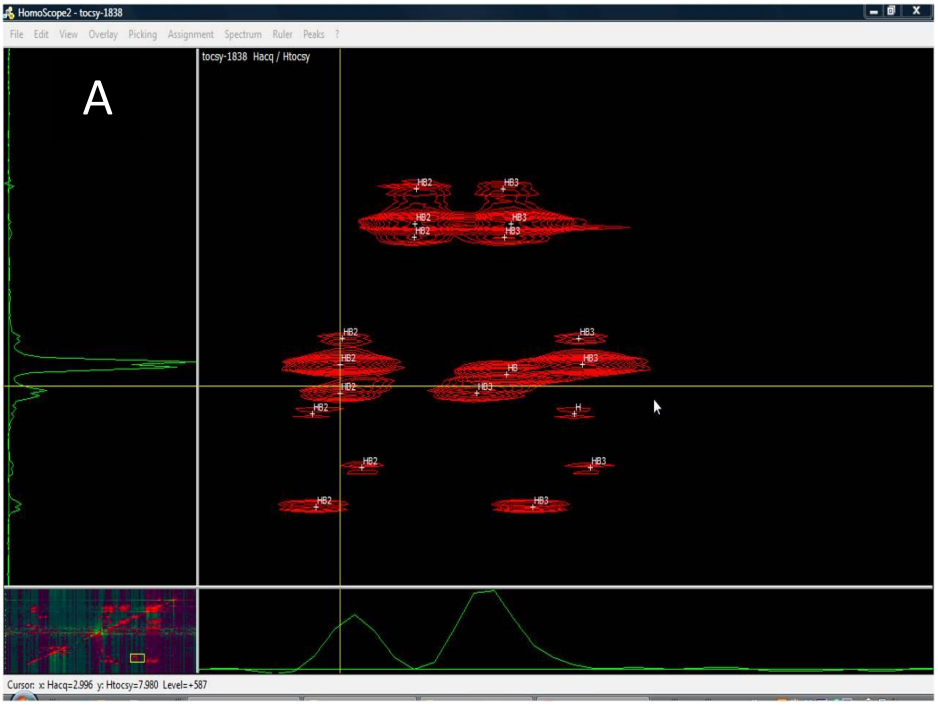


Figure 3. HPLC profiles of P31-43 and three of its alanine scanning mutants

NMR analyses in water confirmed the presence of multiple conformers with inter-conversion rates from slow to fast. For P31-43, Ala6 and Ala7 analogues, spectral overlapping hindered to confidently identify all the components of the molecular ensembles.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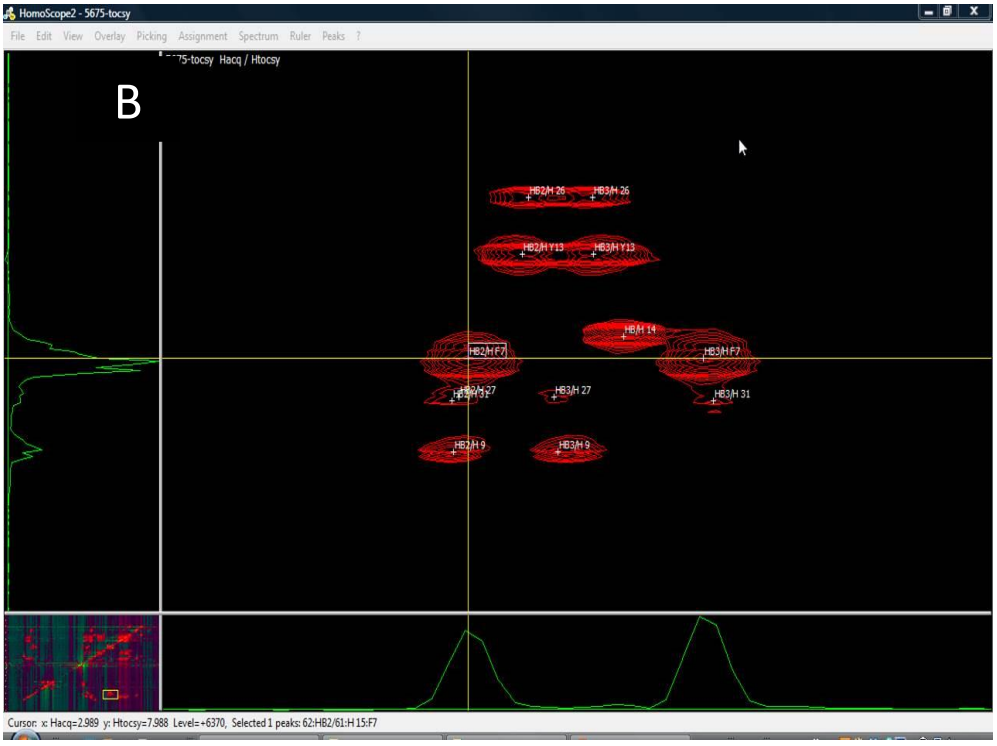
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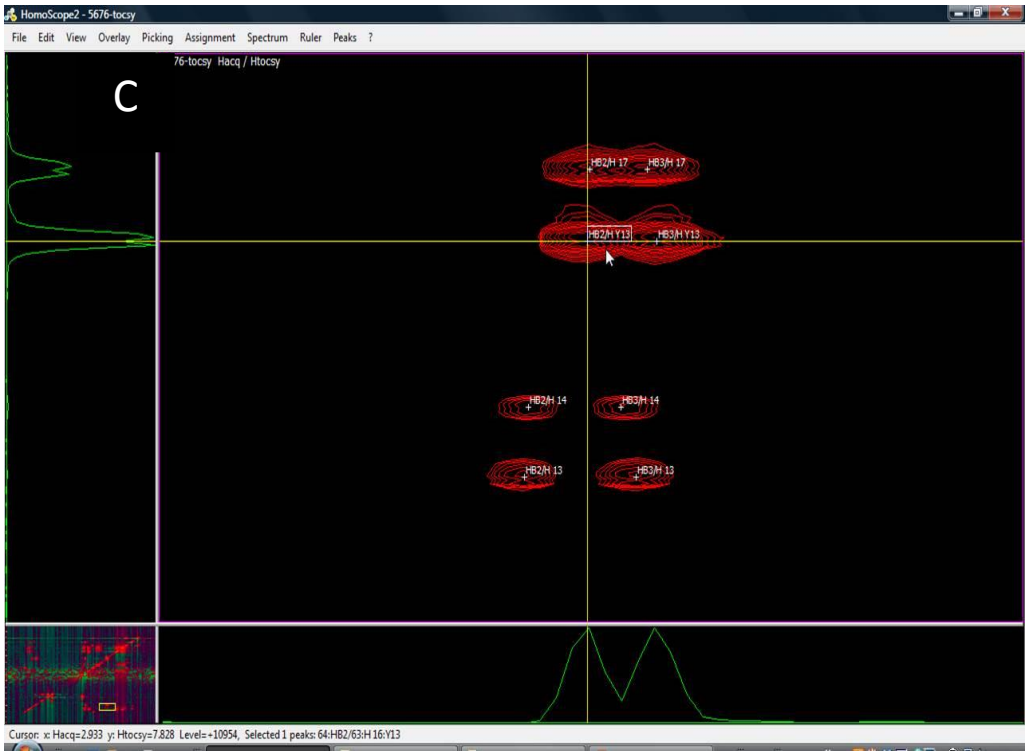

Figure 4. NMR analyses of P31-43(A), Ala6(B), Ala7(C)

However, the structure characterized by P6,P8,P9,P12 in trans configuration was probably the most represented in solution (Figure 5).

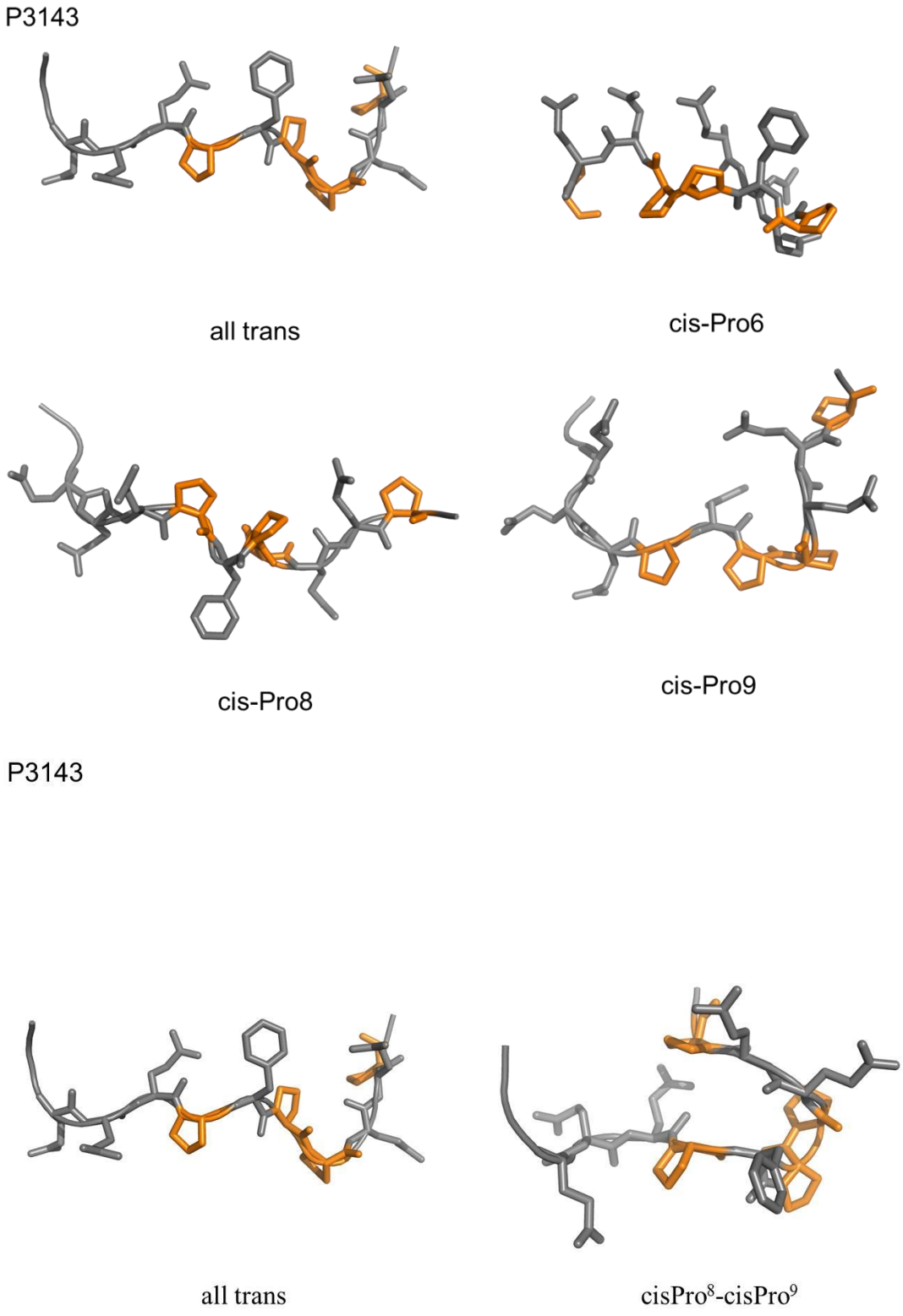

Figure 5. Structure representation of P31-43 in solution

1.2 Effects of Ala-mutant on cell culture CaCo2

EGFR signaling to the nuclei, to induce proliferation, is well characterized (44,45). Upon EGF linking to the EGFR, a signaling cascade starts, that involves sequential phosphorylation of downstream effectors such as ERK. The MAPK-ERK 1–2, like all mitogen-activated-kinases (MAPKs), is one of the essential signaling molecules that convert environmental inputs into influences on a plethora of cellular programs, including proliferation (46). Moreover, most of the MAPK, including ERK, are stress sensors that can be activated by different inputs (47). Only phosphorylated ERK can transduce to the nuclei, where it can start trans-activation of several genes that can induce cell proliferation and other biological effects (48,49).

To date gliadin peptides and in particular P31-43 are able to enhance proliferation of celiac enterocytes in a EGFR/ERK dependent way and to delay the trafficking and degradation of EGFR at the epithelial level, suggesting a role of EGFR activation in CD, particularly in determining the crypts hyperplasia and the tissue remodelling of the CD intestine (38). Moreover P31-43 is also able to reduce TEER in CaCo2 layers.

In this study we have tested the Ala-mutants of P31-43 to identification the critical amino acid residues of P31-43 that can induced tissue damage.

We used cell culture CaCo2 and we evaluated ERK phosphorylation and TEER.

Interesting, eleven of thirteen Ala mutant are able to increase ERK phosphorylation similar to P31-43, but two peptide, Ala6 and Ala7, not induced this increase (Figure 5).

We tested Ala6 and Ala7 and one, Ala13, that have the same effect on ERK phosphorylation like P31-43, on a CaCo2 monolayer, to investigate their effects on intestinal integrity. Interesting Ala 13 is able to reduce TEER of caco2 layers like to P31-43, instead two Ala-mutant, Ala6 and Ala7 are similar to the untreated.

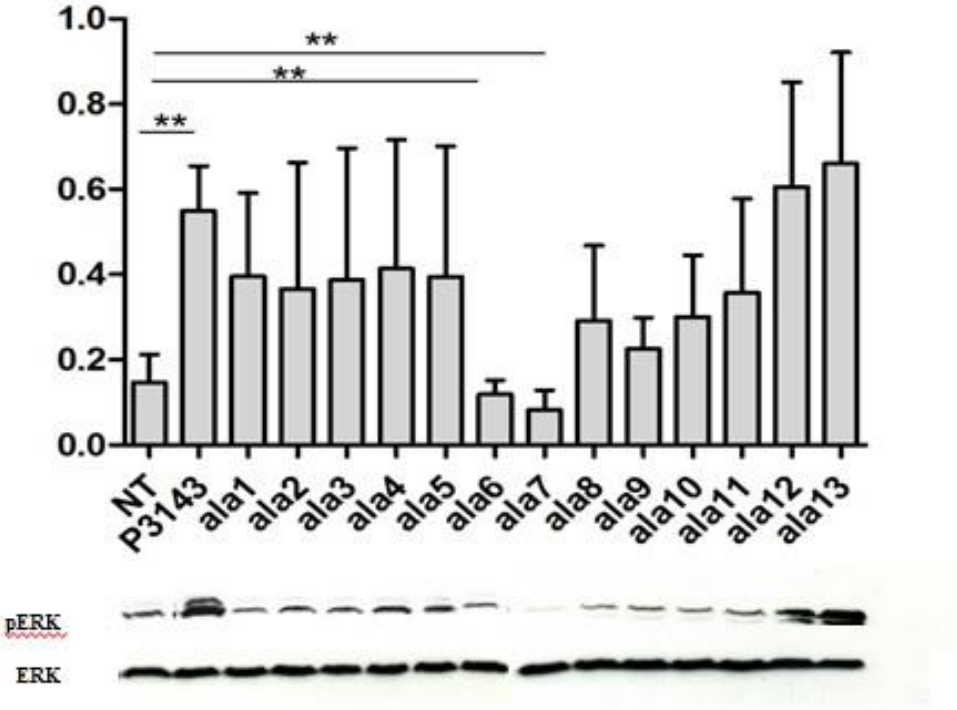

Figure 6. Western blot analysis revealed that P3143 increase phosphorylation of ERK compared two Ala-mutant, Ala6 and Ala7.

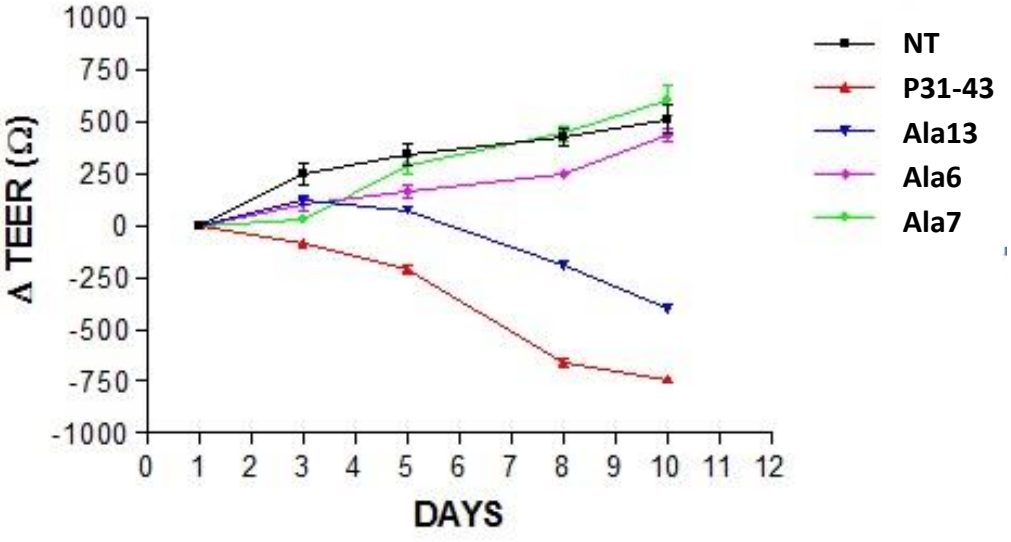

Figure 7. P31-43 is able to reduce TEER of caco2 layers, instead two Ala-mutant, Ala6 and Ala7 are similar to the untreated

Conclusive remarks

Gluten is a complex mixture of polypeptides. The main immunogenic peptides of gluten belong to a family of closely related proline and glutamine-rich proteins called prolamines (15% proline and 35% glutamine residues). CD is triggered by peptides that result from the fragmentation of prolamines, and are not digested by human proteases because the high proline and glutamine content prevents complete proteolysis by gastric and pancreatic enzymes, and long oligopeptides that are toxic to coeliac sprue patients build up in the small intestine.In vitro and in vivo studies have demonstrated that a 25-mer, P31-43, peptide from gliadin is not digestible by gastric, pancreatic, and intestinal brush-border membrane endoproteases. This and other peptides have been identified as the principal contributors to gluten immunotoxicity.

It has been demonstrated that P31-43 is able to increase ERK phosphorylation and to decrease TEER on CaCo2 cells. In the present study we performed Alanin-scanning of P31-43 and tested its mutant on CaCo2 cells.

We observed that Ala mutant of P31-43, in particular, Ala 6 and Ala 7 peptides, not induced this toxic effects on CaCo2 cells. We also observed that the replacement of amino acid residues in position 6 and 7 induces a conformational change of the peptides, as showed by HPLC profiles. The absence of toxic activity of the latter may depend both on the conformational change or the specific amino acid residues. Further experiments are needed to test the harmless of these two peptides using other biological system as the system of organ culture. If confirmed these data could lead to search grains in which this amino acid changes are still present and they could be tested in celiac patient.

2. Autoimmunity in potential CD patient

Gluten is able to induce the production of autoantibodies that can be found in the serum of CD patients and that have, from a diagnostic viewpoint, a very high sensitivity and specificity (50). These autoantibodies recognize an endomysial antigen known today as transglutaminase 2 (TG2) (51). It is also well known that in CD patients anti-TG2 autoantibodies are produced at intestinal level (52), and can be deposited on the extracellular TG2 in the mucosa of small intestine (53), even before passing into circulation and being measurable in serum. These deposits are localized below the basement membrane, along the villous and crypt and around mucosal vessels (53). In our paediatric population, deposits of immunoglobulin (Ig)A anti-TG2 are present in 96% of coeliac patients with overt disease (54). Patients with serum positivity for anti-TG2 and normal duodenal mucosa, or with slight signs of inflammation, are defined today as potential coeliac patients (55). In this group of patients intestinal deposits of IgA anti-TG2 are found less often (54,56). However, their presence is relevant, as it has been associated with the risk of developing frank villous atrophy (56). Several studies have reported that when specimens of duodenal mucosa are cultured for 24–48 h in medium alone and with peptic–tryptic digest of gliadin (PTG) (57,58) or with peptide 31–43 (P31–43) (58,59) , there is a production of anti-endomysial antibodies in organ culture supernatants in CD patients, but not in controls.

More recently, similar data have been obtained measuring IgA anti-TG2 antibodies in supernatants of cultured biopsies from CD patients untreated and on a gluten-free diet (60-62). This test has been proposed for diagnostic purposes, particularly in patients with normal mucosa. However, in this regard the information on potential CD patients is poor and scanty.

The aim of our work was to investigate the presence of anti-TG2 intestinal antibodies in patients with potential CD, and identify the most suitable detection test for this purpose. We compared two assays: the search for intestinal deposits of IgA anti-TG2 and measurement of the same antibodies in the supernatants after organ culture of duodenal biopsies. We detected mucosal deposits of anti-TG2 antibodies in 68% of potential CD patients, confirming our previous findings (54) ; at the same time, we showed in the same patients that a higher number (96%) secreted these antibodies into culture supernatants. The titres of secreted anti-TG2 antibodies of potential CD patients were lower than those observed in active CD patients and correlated significantly with serum titres. It is likely that a correlation exists between intestinal antibody titres and severity of mucosal damage. In our hands, the assay showed a sensitivity and specificity of 97·5% and 92·3%, respectively, differing from the search of mucosal deposits that showed sensitivity and specificity of 77·5% and 80%, respectively, in the same population. Our data also showed that gliadin peptides do not influence intestinal anti-TG2 antibody production in patients with active CD and, as expected, in control subjects.Moreover, the intensity of mucosal deposits seemed to increase in potential CD patients in response to culture with gliadin peptides, while in the majority of patients the titres of secreted autoantibodies did not show a substantial increase.We have demonstrated that to detect intestinal anti-TG2 antibodies, the sensitivity and specificity of the anti-TG2 supernatant are higher than those of mucosal deposits. This test could prove useful in clinical practice to predict evolution to mucosal atrophy in potential coeliac patients and to identify patients with gluten sensitivity who lack CD-associated serum autoantibodies.

These data have been published as Article on *Clinical Experimental Immunology*, for the manuscript see below.

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The Journal of Translational Immunology

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Intestinal anti-tissue transglutaminase antibodies in potential coeliac disease

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Summary

Anti-tissue transglutaminase 2 (anti-TG2) antibodies are present in the serum of the great majority of untreated coeliac disease (CD) patients. They are produced and deposited in the small intestinal mucosa. Potential CD patients present serum anti-TG2 antibodies higher than cut-off, but a normal duodenal mucosa where mucosal deposits of anti-TG2 are not always detectable. The aim of our work was to investigate the presence of anti-TG2 intestinal antibodies in patients with potential CD, and identify the most sensitive test to detect them. Twelve active CD patients, 28 potential CD patients and 39 non-CD controls were enrolled. Biopsy fragments from all patients were analysed by double immunofluorescence to detect mucosal deposits of anti-TG2 antibodies. Fragments from the same subjects were also cultured for 24 h with medium in the presence or absence of gliadin peptides. Anti-TG2 autoantibodies secreted into supernatants were measured by enzyme-linked immunosorbent assay. All active CD, 68% of potential CD patients and 20% of non-CD controls showed mucosal deposits of immunoglobulin (Ig)A anti-TG2; at the same time 100, 96 and 8% of active CD, potential CD and non-CD control patients secreted these antibodies in culture supernatants, respectively. Our data showed that, to detect intestinal anti-TG2 antibodies, the measurement of antibodies secreted into culture supernatants has higher sensitivity and specificity (97·5 and 92·3%, respectively) than the detection of mucosal deposits (77·5 and 80·0%, respectively). The measurement of intestinal anti-TG2 antibodies may prove useful in clinical practice to predict evolution towards mucosal atrophy in potential coeliac patients and identify patients with gluten sensitivity.

Keywords: anti-tissue transglutaminase2, gluten sensitivity, intestinal deposits, potential coeliac disease, secreted antibodies

Introduction

Coeliac disease (CD) is an immune-mediated systemic disorder elicited, in genetically susceptible individuals, by wheat gluten and related proteins of barley and rye [1]. CD is characterized by the presence of a variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, human leucocyte antigen (HLA)-DQ2 and DQ8 haplotypes and enteropathy [2]. It is now clear that, as well as a spectrum of clinical presentations, there is a wide spectrum of alterations of the jejunal histology, ranging from an infiltrative lesion to villous atrophy [3]. Gluten is able to induce the production of autoantibodies that can be found in the serum of CD patients and that have, from a diagnostic viewpoint, a very high sensitivity and specificity [4]. These autoantibodies recognize an endomysial antigen known today as transglutaminase 2 (TG2) [5]. It is also well known that in CD patients anti-TG2 autoantibodies are produced at intestinal level [6], and can be deposited on the extracellular TG2 in the mucosa of small intestine [7], even before passing into circulation and being measurable in serum. These deposits are localized below the basement membrane, along the villous and crypt and around mucosal vessels [7]. In our paediatric population, deposits of immunoglobulin (Ig)A anti-TG2 are present in 96% of coeliac patients with overt disease [8].

Patients with serum positivity for anti-TG2 and normal duodenal mucosa, or with slight signs of inflammation, are defined today as potential coeliac patients [2,9]. In this group of patients intestinal deposits of IgA anti-TG2 are found less often [8,10]. However, their presence is relevant, as it has been associated with the risk of developing frank villous atrophy [10].

Several studies have reported that when specimens of duodenal mucosa are cultured for 24–48 h in medium alone and with peptic–tryptic digest of gliadin (PTG) [11,12] or with peptide 31–43 (P31–43) [13,14], there is a production of anti-endomysial antibodies in organ culture supernatants in CD patients, but not in controls.

More recently, similar data have been obtained measuring IgA anti-TG2 antibodies in supernatants of cultured biopsies from CD patients untreated and on a gluten-free diet [15–17]. This test has been proposed for diagnostic purposes, particularly in patients with normal mucosa. However, in this regard the information on potential CD patients is poor and scanty.

The aim of our work was to investigate the presence of anti-TG2 intestinal antibodies in patients with potential CD, and identify the most suitable detection test for this purpose. We compared two assays: the search for intestinal deposits of IgA anti-TG2 and measurement of the same antibodies in the supernatants after organ culture of duodenal biopsies.

Patients and methods

Patients

Our study involved 40 patients (29 females, median age 7 years, range 2–17 years) who underwent a small intestinal biopsy at the Department of Pediatrics, University Federico II in Naples for suspicion of CD. All patients presented increased serum levels of anti-TG2 antibodies. Twelve patients showed total villous atrophy (Marsh classification grade IIIc) and received a diagnosis of CD on the basis of diagnostic criteria established by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) [2]. Twenty-eight of 40 patients showed architecturally normal small intestinal mucosa (11 with grade 0 and 17 with Marsh classification grade I); they were classified as potential CD patients. Thirty-nine subjects without coeliac disease (22 females, median age 8 years, range 1–16 years) were enrolled as a control group. All non-CD patients presented normal serum levels of anti-TG2 antibodies and architecturally normal small intestinal mucosa (30 with grade 0 and 9 with Marsh classification grade I). Twelve of 39 subjects had type 1 diabetes (T1D). The final diagnoses of the remaining 27 subjects were iron deficiency anaemia, failure to thrive, gastroesophageal reflux and recurrent abdominal pain. IgA deficiency was excluded.

Written informed consent was obtained by the parents of the children enrolled. The study protocol was approved by the Ethical Committee of the University 'Federico II' Naples, Italy (CE 230/05).

Duodenal biopsy and organ culture system

During upper gastrointestinal endoscopy, at least five duodenal biopsies were taken from all patients. Two fragments were fixed in 10% formalin, embedded in paraffin, and then treated for histological analysis. One of the duodenal specimens was embedded in an optimal cutting temperature compound (OCT; Tissue-Tek, Sakura Finetek Europe BV, Zoeterwoude, the Netherlands) and stored in liquid nitrogen until used. The remaining two fragments were cultured for 24 h at 37°C in either the presence or absence of P31–43 (0·1 mg/ml)/peptic–tryptic gliadin digest (PTG; 0·5 mg/ml). Organ culture was performed as reported previously [18]. After 24 h of culture, the tissues were embedded in OCT and stored in liquid nitrogen. The culture supernatants were collected and stored at −80°C until they were analysed.

Intestinal deposits of anti-TG2 IgA antibodies

The presence of intestinal deposits of anti-TG2 IgA was investigated before and after 24 h of organ culture with P31–43 or PTG or medium alone. Five μm cryostat sections were stained using a double-immunofluorescence method, as described previously [19]. The stained sections were evaluated using a confocal microscope (LSM510; Zeiss MicroImaging Inc., Milan, Italy).

Measurement of anti-TG2 IgA antibodies secreted into culture supernatants

Mucosal anti-TG2 IgA antibodies secreted into culture supernatants were measured in undiluted supernatants by enzyme-linked immunosorbent assay (ELISA; EU-tTG IgA kit; Eurospital S.p.A, Trieste, Italy), according to the manufacturer's instructions. When the value of anti-TG2 was higher than the last point of standard curve, supernatants were diluted 1:2, 1:4, 1:10 and 1:20 in culture medium. The cut-off value for anti-TG2 IgA antibodies in culture supernatants was calculated using supernatants from cultures with medium alone from the 39 controls and was established as the mean ± 2 standard deviations (SD) (2·8 U/ml).

Statistics

Statistical analysis was performed using GraphPad Prism 4 for Windows, version 4·03. Data with a Gaussian distribution were compared by Student's *t*-test; Pearson's χ^2 test was used for non-normal data; Pearsons' correlation test was used to compare titres of anti-TG2 IgA in serum and supernatants in potential CD patients. A *P*-value of < 0.05 was

considered to be significant. The sensitivity and specificity of the two methods examined were calculated by standard statistical formulae.

Results

Intestinal deposits of anti-TG2 IgA

Mucosal deposits of anti-TG2 IgA were searched in small intestinal fragments from 12 untreated CD, 28 potential CD and 30 control patients. All CD patients (100%) in the active phase of disease, 19 of 28 (67·8%) potential CD and six of 30 (20%) non-CD patients were positive. Among the six positive non-CD subjects, five were T1D patients and one affected by gastroesophageal reflux. In most potential positive patients and in the six positive controls a patchy distribution of mucosal deposits was observed. The value of serum anti-TG2 in those potential CD patients (nine of 28) without deposits ranged from 7 to 20·4 U/ml. Moreover, we confirmed that titres of serum anti-TG2 were statistically lower in patients negative for intestinal deposits (mean ± SD: 13·6 ± 5·0 U/ml) than in those positive (29·8 ± 30·3 U/ml, $P < 0.05$), as shown previously [10].

We also looked for the presence of intestinal deposits in duodenal fragments after 24 h of *in vitro* challenge with P31–43 or PTG, to investigate if gliadin peptides were able to induce the production and consequently the deposition of anti-TG2 antibodies. Culture experiments were performed with duodenal fragments from four of 12 untreated CD (one cultured in the presence of P31–43 and three in the presence of PTG), 20 of 28 potential CD (16 with P31–43 and four with PTG) and 14 of 30 control patients (11 with P31–43 and three with PTG) (Table 1). We found no significant change in the intensity of mucosal IgA deposits in biopsies from active CD cultured with P31–43/PTG when compared to those cultured with medium alone or in samples before culture. In 15 of 20 (75%) potential CD patients a higher intensity of mucosal IgA deposits was seen in fragments cultured with P31–43/PTG if compared to medium alone. Moreover, in fragments cultured with P31–43, 18 of 20 (90%) patients showed mucosal deposits, compared to 12 of 20 (60%; $P < 0.02$) patients in fragments cultured in medium alone (Fig. 1). Our data also showed that after 24 h culture with medium alone a lower intensity of mucosal deposits was seen in eight of 20 (40%) potential CD patients compared to what was seen before culture.

IgA anti-TG2 in organ culture supernatants

We investigated the presence of anti-TG2 IgA in supernatants obtained after 24-h culture of biopsies from 12 active CD, 28 potential CD and 39 non-CD patients (Table 1). Anti-TG2 IgA antibodies in supernatants of biopsies cultured with medium only were higher than cut-off in 12 of 12 (100%) active CD, 27 of 28 (96·4%) potential CD and three of 39 (7·6%) non-CD patients. All active CD patients secreted very high titres, ranging from 287·2 to 2020·3 U/ml. Titres of secreted anti-TG2 in potential CD patients were variable, ranging from 5·75 to 1005 U/ml (Fig. 2), and were correlated with serum anti-TG2 titres (Pearson's $r = 0.68$, $P < 0.0001$) (Fig. 3). Of the three positive non-CD patients, one was a first-degree relative, the second was affected by T1D and the third was affected by gastroesophageal reflux disease (GERD) and headache. They secreted low amounts of anti-TG2 (range 3·33–5·02 U/ml). Among these three non-CD patients the detection of mucosal deposits of anti-TG2 IgA antibodies in fragments before culture was positive in only one of three (the T1D patient).

In cultured supernatants of all active CD patients after 24 h P31–43 or PTG *in vitro* stimulation, anti-TG2 antibodies titres were comparable to those of medium alone. Potential CD patients showed a wide spectrum of responses after P31–43/PTG *in vitro* challenge: the titres of supernatants anti-TG2 IgA were reduced or stable in six of 28 and 16 of 28, respectively; we found a more than doubled increase in only six of 28 patients. Among controls, all 36 patients who were negative for production of anti-TG2 antibodies in medium alone confirmed their negativity even after stimulation with P31–43/PTG; two of three control patients positive for secreted antibodies into medium culture were also positive after PTG stimulation with levels of anti-TG2 IgA comparable to those of medium alone.

The measurement of anti-TG2 titres in culture supernatants seems to be more sensitive and specific than the detection of mucosal deposits to reveal mucosal production of anti-TG2 antibodies in coeliac disease, showing a sensitivity and specificity of 97·5 and 92·3% *versus* 77·5 and 80% of mucosal deposits, respectively. Moreover, the rate of

Table 1. Detection of mucosal deposits and measurement of supernatant anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A in the study population.

	Fragments showing IgA anti-TG2 mucosal deposits					Supernatants with anti-TG2 higher than cut-off*		
Patients	*n*	Before culture	*n*	After medium 24 h	After P31–43/PTG 24 h	*n*	Medium	P31–43/PTG
Active CD	12	12/12	4	4/4	4/4	12	12/12	12/12
Potential CD	28	19/28	20	12/20	18/20	28	27/28	26/28
Non-CD	30	6/30	14	1/14	5/14	39	3/39	2/39

*Cut-off = 2·8 U/ml. CD: coeliac disease; PTG: peptic–tryptic digest of gliadin.

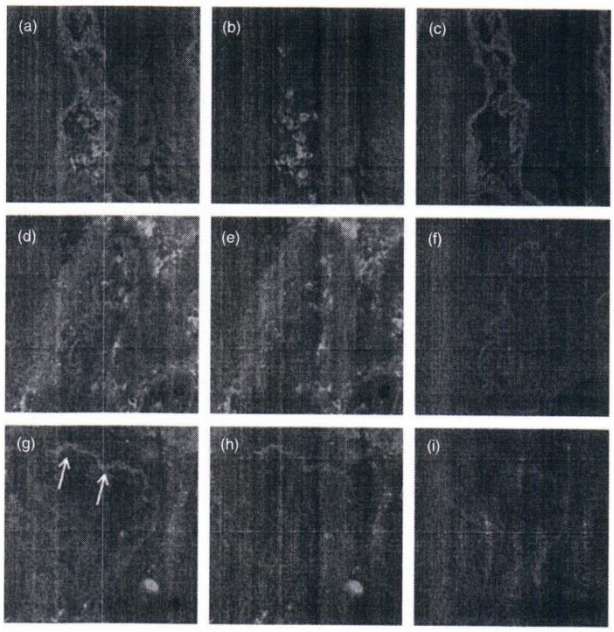

Fig. 1. Duodenal mucosa from potential coeliac disease (CD) patient negative for mucosal deposits of immunoglobulin (Ig)A anti-transglutaminase 2 (TG2) before (a–c) and after 24 h culture with medium alone (d–f). Mucosal deposits of IgA anti-TG2 (in yellow) is lightly visible after 24-h culture with P31–43 (arrows) (g–i). IgA secreted by plasma cells are visible in green (b,e,h); TG2 with a subepithelial localization is shown in red (c,f,i).

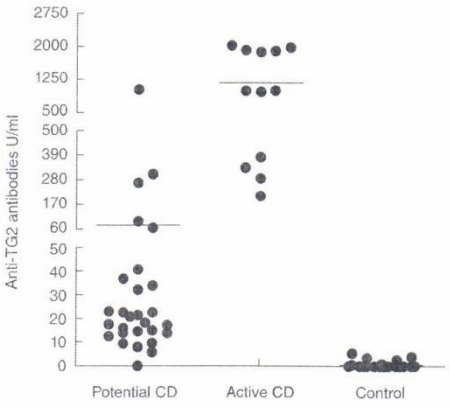

Fig. 2. Titres of anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A, expressed as U/ml, in medium culture supernatants of all culture experiments. Horizontal lines represent mean values.

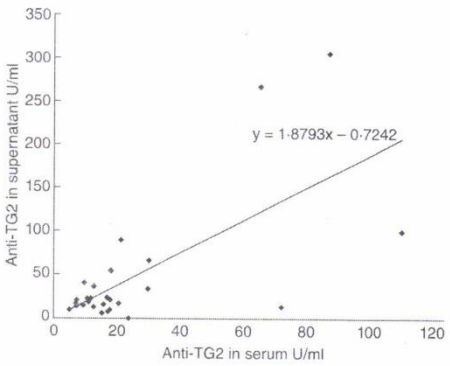

Fig. 3. Correlation between titres of anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A (expressed as U/ml) in serum and in culture supernatants of intestinal fragments from 28 potential coeliac disease (CD) patients. Equation for the line: $y = 1{\cdot}8793x - 0{\cdot}7242$ ($R^2 = 0{\cdot}4596$).

concordance of the two methods compared with serum anti-TG2 autoantibodies was 78·6% for intestinal deposits and 94·9% for supernatants anti-TG2, respectively ($P < 0.01$).

Discussion

The presence of anti-TG2 antibodies in serum represents a CD marker [4]. However, intestinal anti-TG2 antibodies may be even more relevant from a clinical viewpoint. These antibodies are produced at intestinal level [6], where they could be deposited even before they appear in circulation [7,20]. Two aspects can make intestinal anti-TG2 antibodies relevant: the first is their suggested ability in potential coeliac patients to predict evolution towards a clear enteropathy; the second is their possible role in revealing a condition of gluten sensitivity in patients with absence of CD-associated autoantibodies in their serum [21,22]. Therefore, it is particularly important to find the most suitable assay to detect and measure these antibodies.

In the last 20 years different assays have been used: measurement in faeces [23] or in intestinal fluids [24], search in supernatants of cultured biopsy fragments [11–17] or detection in the same biopsies of deposited antibodies [16], or expression in phage libraries of RNA, obtained from biopsies, coding for the antibodies [6,22]. Detection of anti-endomysium antibodies (EMA) and anti-TG2 IgA antibodies in faecal supernatants from CD patients was determined to be unreliable as a diagnostic test [25]. The search for anti-TG2 antibodies in culture supernatants of intestinal biopsy from CD patients by ELISA was proposed in particular to improve the accuracy of CD diagnosis in subjects with mild enteropathy and in patients negative for serum antibodies [15–17]. More recently, using immunofluorescence, Korponay-Szabo *et al.* [7] showed that IgA deposited in the intestine of CD patients are directed against TG2. This assay, while having high sensitivity and specificity for the diagnosis, requires frozen intestinal samples and highly experienced operators. By means of phage display libraries, Marzari *et al.* [6] showed that anti-TG2 IgA antibodies are synthesized primarily by specific B lymphocytes in the small intestinal mucosa and that there is a preferential use of heavy chain variable regions belonging to the VH5 gene family in antibodies from coeliac patients. The intestinal production of anti-TG2 antibodies has been confirmed recently by the finding of a high abundance of plasma cells secreting TG2-specific IgA autoantibodies with limited somatic hypermutation in CD intestinal lesions [26]. In identifying patients with gluten sensitivity, the phage display libraries technique seems to be more sensitive than detection of mucosal deposits of anti-TG2 antibodies using double immunofluorescence assay [22]. At the same time, it must be emphasized that, because of its complexity, this test cannot be proposed for routine use.

In this study, not using faeces detection and considering the phage library technique too demanding, we compared the diagnostic efficiency of the detection of intestinal deposits of these antibodies and their measurement in biopsy culture supernatants, particularly in patients with potential CD. We detected mucosal deposits of anti-TG2 antibodies in 68% of potential CD patients, confirming our previous findings [8]; at the same time, we showed in the same patients that a higher number (96%) secreted these antibodies into culture supernatants. The titres of secreted anti-TG2 antibodies of potential CD patients were lower than those observed in active CD patients and correlated significantly with serum titres. It is likely that a correlation exists between intestinal antibody titres and severity of mucosal damage. In our hands, the assay showed a sensitivity and specificity of 97·5% and 92·3%, respectively, differing from the search of mucosal deposits that showed sensitivity and specificity of 77·5% and 80%, respectively, in the same population. Furthermore, the measurement of anti-TG2 antibodies secreted into culture supernatants is performed by ELISA, so it is objective and not influenced by the operator's ability for the detection of mucosal deposits.

Our data also showed that gliadin peptides do not influence intestinal anti-TG2 antibody production in patients with active CD and, as expected, in control subjects. Moreover, the intensity of mucosal deposits seemed to increase in potential CD patients in response to culture with gliadin peptides, while in the majority of patients the titres of secreted autoantibodies did not show a substantial increase. Stimulation of specific B lymphocytes could induce production of the anti-TG2 antibodies that bind the tissue-transglutaminase and increase deposits signalling without increasing the titres in culture supernatants.

As mentioned previously, the clinical relevance of detecting intestinal anti-TG2 antibodies resides in their ability to predict evolution to mucosal damage and in identifying subjects with gluten sensitivity. In the first case, the presence of intestinal deposits of anti-TG2 seems to be a marker of forthcoming overt CD [10,27] in patients with normal intestinal mucosa. This ability, if confirmed by larger studies, could help to solve the vexed question of whether or not potential CD patients must be put onto a gluten-free diet or remain on a gluten-containing diet. Currently, our centre's policy is to leave asymptomatic potential CD patients on a gluten-containing diet and follow them over time. With regard to the state of gluten sensitivity, important observations have been made measuring antibodies in intestinal fluids and using the phage library technique. Using the first approach, patients with gluten sensitivity were identified among patients with irritable bowel syndrome who presented these antibodies in intestinal fluids, but not in serum [28]. Recently, Not *et al.* [22] have reported that a large proportion of DQ2/DQ8-positive relatives of CD patients with normal mucosa and without measurable serum levels of anti-TG2 produce these

antibodies in the intestine in response to gluten. We showed that two of the three patients in the control group who secreted anti-TG2 into culture supernatants were, in one case, a first-degree relative and in the second case a patient affected by type 1 diabetes. This last finding is not surprising, as we have already demonstrated that patients with type 1 diabetes, without serum anti-TG2 and with normal duodenal mucosa, produce and deposit anti-TG2 antibodies in their small intestinal mucosa [29]. Finally, in the third case, there was little clinical evidence to support a condition of non-coeliac gluten sensitivity (NCGS). All the patients had normal anti-gliadin levels. So far, this is the only candidate marker of such a condition [30]. In this context, the presence of intestinal autoantibodies against TG2 should be assessed.

In conclusion, in this work we have demonstrated that to detect intestinal anti-TG2 antibodies, the sensitivity and specificity of the anti-TG2 supernatant are higher than those of mucosal deposits. This test could prove useful in clinical practice to predict evolution to mucosal atrophy in potential coeliac patients and to identify patients with gluten sensitivity who lack CD-associated serum autoantibodies.

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Disclosure

The authors have no conflicts of interest.

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3. Immunoregulatory pathway in potential CD patient

The characteristic features of CD histology have always been considered to be a villous atrophy, crypt cell hyperplasia, and increased number of intraepithelial lymphocytes (IELs) (63). However, more recently, it has become increasingly evident that CD is not restricted to severe gluten-dependent enteropathy. Since 1993, Ferguson et al. (64) proposed the expression of potential CD to indicate subjects with a normal, or almost normal jejunal mucosa, but with immunological abnormalities similar to those found in celiac patients (64). Recently, this condition has been redefined and related to people with a normal small intestinal mucosa who are at increased risk to developing CD, as indicated by positive CD serology (65).

Although the pathogenesis of CD remains not fully unraveled, it is known that gluten peptides are deamidated by tissue transglutaminase and presented by HLA-DQ2+ or HLA-DQ8+ antigen-presenting cells to lamina propria CD4+ T cells (65,66). Upon activation, the lamina propria CD4+ cells polarize along the T helper 1 (Th1)-type pathway, as substantiated by their ability to produce large amounts of interferon γ (IFNγ), the main cytokine of Th1 response (67,68). In untreated celiac mucosa, the production of IFNγ is massively increased and its secretion can be observed in organ cultures from CD patients on a gluten-free diet, and in lamina propria derived T-cell clones upon stimulation with gliadin peptides (68-70). However, beside the adaptive immunity, the innate immune response is necessary for the phenotypic expression and pathologic changes characteristic of CD (71). Interleukin-15 (IL-15), a potent pro-inflammatory cytokine upregulated in the intestinal epithelium of CD, is thought to have pleiotropic function at the interface between innate and adaptive immunity (72,73).

At the same time, an enhanced expression of anti-inflammatory cytokines, such as IL-10, has also been observed in CD. In fact, in untreated CD patients the levels of IL-10 are higher, but the ratio IL-10/IFNγ is significantly lower in comparison with inflamed non-celiac, control, and treated CD mucosa (74,75). Interestingly, the number of CD4+CD25+ T regulatory cells that express the forkhead box P3 transcription factor (Foxp3+) (Foxp3+Tregs) is significantly increased in the small intestinal mucosa with active CD (76,77). Furthermore, our recent studies have revealed that in active CD Foxp3+Tregs are functionally active, but their suppressive capacity might be impaired by pro-inflammatory cytokines, such as IL-15 (77).

Also potential CD shows markers of enhanced inflammatory reactions as shown by the high level of IFNγ mRNA and densities of tumor necrosis factor-α-positive cells in the lamina propria (78); at the same time, in this condition the number of FoxP3-expressing cells is higher (79).

Evidence suggests that CD develops gradually from normal villous architecture with only an epithelial lymphocytosis, through partial to total villous atrophy. The innate and adaptive mucosal systems are tightly controlled by various regulatory circuits, and it is possible that defects in such mechanisms could have a role in this dynamic process of mucosal damage in CD. In this respect, we think that potential CD represents an early stage of disease, and accordingly a good condition to clarify the early immunological events implicated in the onset of CD. For this purpose, initially we investigated in duodenal biopsies from potential CD subjects the state of immunological activation, with analysis of cytokines involved in both innate and adaptive immune responses, such as IL-15 and IFNγ. Thereafter, to clarify the role of immunoregulatory mechanisms, we evaluated the expression of anti-inflammatory cytokines and the number of Foxp3+Tregs.

Finally, we evaluated the functional capacity of intestinal Foxp3+Tregs, obtained from potential CD patients and the role of IL-15 on their suppressive function.

We provide evidence that in potential CD, despite the absence of mucosal damage, there are clear signs of infl ammation; in particular, our data suggest that in duodenal mucosa of these patients, T cells seem to be activated and diff erentiating toward a Th 1 pattern. Indeed, IL-2 RNA expression is increased in the mucosa of potential CD when compared with controls and active celiacs, confirming our previous data (80). Also, IFN γ -RNA expression, as well as the percentage of IFN γ + CD4 + cells, is significantly increased. Interestingly, IFN γ levels correlate with IEL infiltration, in agreement with previous data showing that the majority of IFN γ is produced in the epithelium (75)

Our data suggest that regulatory mechanisms have a crucial role to downregulate the inflammation in early phase of CD. We have shown that in this condition there are two factors that maintain the suppressive action of Foxp3 + Treg: they are the high IL-10 expression and a low sensitivity to pro-inflammatory stimuli, such as IL-15.

These data have been published as Article on *The American Journal of Gastrenterology*,

for the manuscript see below.

Immunoregulatory Pathways Are Active in the Small Intestinal Mucosa of Patients with Potential Celiac Disease

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OBJECTIVES: **Potential celiac disease (CD) relates to subjects with a normal small intestinal mucosa who are at increased risk of developing CD as indicated by positive CD-associated serology. The objective of this study was to investigate in the small intestinal mucosa of such patients the state of immunological activation with special emphasis on immunoregulatory circuits.**

METHODS: **Duodenal biopsies from active CD ($n=48$), potential CD ($n=58$), and control patients ($n=45$) were studied. RNA expression for interferon γ (IFNγ) and interleukin-10 (IL-10) were quantified by real-time quantitative PCR. The percentage of CD4+CD25+Foxp3+ T regulatory cells (Foxp3+Tregs) was determinated by flow cytometry and the number of Foxp3+ and IL-15+ cells by immunohistochemistry. Furthermore, we analyzed the suppressive function of CD4+CD25+ T cells, isolated from potential CD biopsy samples, as well as the effect of IL-15, on autologous peripheral blood responder CD4+CD25− T cells.**

RESULTS: **In potential CD patients with Marsh 1 lesion, IFNγ-RNA expression was significantly less than in active, but enhanced if compared with potential CD patients with Marsh 0 lesion and with controls ($P<0.001$). The number of IL-15+ cells in subjects with potential CD was increased in comparison with controls ($P<0.05$), but lower than active CD ($P<0.01$). IL-10-RNA expression was upregulated in Marsh 0 potential CD patients if compared with those with Marsh 1 lesion ($P<0.01$) and controls ($P<0.001$), whereas there were no differences with active CD. The ratio IL-10/IFNγ reached the highest value in Marsh 0 potential CD compared with the other groups ($P<0.05$). The percentage of Foxp3+Tregs was also higher in potential CD compared with controls ($P<0.05$), although it was lower than in active CD ($P<0.01$). In co-culture assay, intestinal CD4+CD25+ T cells from potential CD patients exerted suppressive effects on T responder cells, and their activity was not impaired by IL-15.**

CONCLUSIONS: **Potential CD patients show a low grade of inflammation that likely could be due to active regulatory mechanisms preventing the progression toward a mucosal damage.**

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/ajg

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INTRODUCTION

Celiac disease (CD) is an immune-mediated systemic disorder elicited by gluten and related prolamines in genetically (mainly HLA) susceptible individuals, characterized by the presence of variable combination of gluten-dependent clinical manifestations, CD-specific antibodies, HLA DQ2 and DQ8 haplotypes, and enteropathy (1). The characteristic features of CD histology have always been considered to

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be a villous atrophy, crypt cell hyperplasia, and increased number of intraepithelial lymphocytes (IELs) (2). However, more recently, it has become increasingly evident that CD is not restricted to severe gluten-dependent enteropathy. Since 1993, Ferguson *et al.* (3) proposed the expression of potential CD to indicate subjects with a normal, or almost normal jejunal mucosa, but with immunological abnormalities similar to those found in celiac patients (3). Recently, this condition has been redefined and related to people with a normal small intestinal mucosa who are at increased risk to developing CD, as indicated by positive CD serology (4).

Although the pathogenesis of CD remains not fully unraveled, it is known that gluten peptides are deamidated by tissue transglutaminase and presented by HLA-DQ2+ or HLA-DQ8+ antigen-presenting cells to *lamina propria* CD4+ T cells (5,6). Upon activation, the *lamina propria* CD4+ cells polarize along the T helper 1 (Th1)-type pathway, as substantiated by their ability to produce large amounts of interferon γ (IFNγ), the main cytokine of Th1 response (7,8). In untreated celiac mucosa, the production of IFNγ is massively increased and its secretion can be observed in organ cultures from CD patients on a gluten-free diet, and in *lamina propria* derived T-cell clones upon stimulation with gliadin peptides (7–9). However, beside the adaptive immunity, the innate immune response is necessary for the phenotypic expression and pathologic changes characteristic of CD (10). Interleukin-15 (IL-15), a potent pro-inflammatory cytokine upregulated in the intestinal epithelium of CD, is thought to have pleiotropic function at the interface between innate and adaptive immunity (11,12).

At the same time, an enhanced expression of anti-inflammatory cytokines, such as IL-10, has also been observed in CD. In fact, in untreated CD patients the levels of IL-10 are higher, but the ratio IL-10/IFNγ is significantly lower in comparison with inflamed non-celiac, control, and treated CD mucosa (13,14). Interestingly, the number of CD4+CD25+ T regulatory cells that express the forkhead box P3 transcription factor (Foxp3+) (Foxp3+Tregs) is significantly increased in the small intestinal mucosa with active CD (15,16). Furthermore, our recent studies have revealed that in active CD Foxp3+Tregs are functionally active, but their suppressive capacity might be impaired by pro-inflammatory cytokines, such as IL-15 (16).

Also potential CD shows markers of enhanced inflammatory reactions as shown by the high level of IFNγ mRNA and densities of tumor necrosis factor-α-positive cells in the *lamina propria* (17); at the same time, in this condition the number of FoxP3-expressing cells is higher (18).

Evidence suggests that CD develops gradually from normal villous architecture with only an epithelial lymphocytosis, through partial to total villous atrophy. The innate and adaptive mucosal systems are tightly controlled by various regulatory circuits, and it is possible that defects in such mechanisms could have a role in this dynamic process of mucosal damage in CD. In this respect, we think that potential CD represents an early stage of disease, and accordingly a good condition to clarify the early immunological events implicated in the onset of CD. For this purpose, initially we investigated in duodenal biopsies from potential CD subjects the state of immunological activation, with analysis of cytokines involved in both innate and adaptive immune responses, such as IL-15 and IFNγ. Thereafter, to clarify the role of immunoregulatory mechanisms, we evaluated the expression of anti-inflammatory cytokines and the number of Foxp3+Tregs. Finally, we evaluated the functional capacity of intestinal Foxp3+Tregs, obtained from potential CD patients and the role of IL-15 on their suppressive function.

METHODS

Patients

We studied pediatric patients with following characteristics: active CD ($n=48$, 27 females, mean age 6. 9 years), having a villous atrophy-type Marsh 3 (2) and high serum levels of anti-human tissue transglutaminase antibodies (anti-TG2), and diagnosed according to ESPGHAN criteria established in 1990 (19); potential CD ($n=58$, 30 females, mean age 6.6 years), characterized by the presence of serum anti-TG2 antibodies, and of HLA-DQ2 and/or HLA-DQ8, but with a normal villous architecture of duodenal mucosa. They were separated in two groups: (i) patients with mucosal lesion-type Marsh 0 ($n=33$) and (ii) Marsh 1 ($n=25$) on the basis of IELs infiltration (Marsh 1 patients had >34/mm IELs). More detailed characteristics of potential CD patients are reported in the Table (see **Supplementary Table 1** online). We also enrolled control patients ($n=45$, 20 females, mean age 6.4 years), who had the following diagnoses: gastroesophageal reflux, *Helicobacter pylori* gastritis, cyclic vomiting syndrome, eosinophilic esophagitis, food allergy, and anorexia. Biopsy specimens from duodenum were obtained by upper gastrointestinal endoscopy. All subjects gave informed consent to the proposed study. The protocol of the study was approved by the Ethical Committee of the University "Federico II" of Naples, Italy (CE115/09).

Real-time quantitative PCR

Total tissue RNA was extracted from whole mucosal biopsies using TRIZOL reagent (Gibco Life Technologies, Milan, Italy), and a constant amount of total RNA (0.5–1 μg) were retrotranscribed into complementary DNA according to the manufacturer's instructions (Invitrogen, Milan, Italy). Real-time quantitative PCR was performed using Icycler iQ Real-Time PCR Detection System (Bio-Rad, Milan, Italy) by TaqMan technologies. TaqMan probes/primers specific for L32, IFNγ, IL-10, IL-2, transcription factor T-bet (T-bet), and interferon regulatory factor 1 (IRF-1) were developed by Bio-Rad. L32 gene RNA was used as an internal control. As a calibrator sample, we used human peripheral blood mononuclear cells stimulated with lypopolisaccharide (1 mg/ml) in RPMI medium for 2 h. The PCR reactions contained 0.2 μM of each primer and 0.2 μM of each probe, Platinum Quantitative PCR SuperMix-UDG buffer (Invitrogen Life Technologies, San Diego, CA) and 5 μl of complementary DNA in a final volume of 50 μl. The quantification of the RNA marker was calculated relatively to the housekeeping L32 gene on the base of comparative $2^{-\Delta\Delta CT}$

method, using a real-time quantification program kindly developed by Bio-Rad.

Flow cytometry

Mucosal explants were digested with collagenase-A and cells were isolated following the method previously described (16). A total of $5–10\times10^4$ cells obtained from fresh biopsies were labeled with fluorochrome-conjugated antibodies: CD4-phycoerythrin (RPA-T4) and CD25-phycoerythrin-Cy5 (M-A251) mAb. Appropriate isotype-matched control antibodies were included in all experiments. In the experiments for intracellular staining, the cells were fixed and permeabilized with cytofix/cytoperm (Becton Dickinson, San Diego, CA) according to the manufacturer's instructions, followed by staining with IL-10-phycoerythrin (JES3-9D7), Foxp3-fluorescein isothiocyanate (PCH101), or anti human IFNγ-fluorescein isothiocyanate antibodies (B27). All the antibodies were provided by BD Pharmingen (San Diego, CA) with the exception of anti-Foxp3 provided by eBioscience (San Diego, CA). Cells were next analyzed by the flow cytometer FACSCalibur with CellQuest Software (Becton Dickinson), in the gate of viable mononuclear cells based on their forward-scatter/side-scatter characteristics.

IL-15 receptor-α surface expression was evaluated on peripheral blood mononuclear cells as previously reported (16).

Immunohistochemistry

For immunohistochemical detection of Foxp3+ cells, cryostat sections (5 μm) were air-dried at room temperature and fixed in acetone for 10 min. All sections were individually incubated for 1 h at room temperature with rat anti-human Foxp3 (1:20; eBioscience) followed by an incubation (40 min) with goat anti-rat biotinilated (1:200; Vector Laboratories, Burlingame, CA), and then with streptavidin-AP (1:100; Dako, Milan, Italy; 40 min), and finally by an incubation with New Fuchsin (Sigma Aldrich, Milan, Italy). Sections were washed thoroughly with tris-buffered saline 0.15 M at each step. All the sections were counterstained with hematoxylin solution (Sigma Aldrich) and mounted with Aquamount (Merck SpA, Milan, Italy). Negative control was performed by omitting the primary antibody (incubation with 1% normal goat serum).

For the detection of IL-15, 5 μm cryostat sections were fixed in 2% formaldehyde in phosphate-buffered saline for 20 min and then treated as previously reported (20).

All the slides were analyzed with a light microscope (Axioskop2 Zeiss, Milan, Italy) by two independent observers blinded to the clinical data. Intra-observer and inter-observer concordance was >95%. The density of cells expressing Foxp3 in the *lamina propria* was evaluated within a total area of 1 mm^2 of *lamina propria* using a microscope with an eyepiece equipped with a graduated reticule aligned parallel to the muscolaris mucosae. IL-15 staining was graded on an arbitrary scale of staining from 1 to 4, as reported in the legend in **Figure 2**.

Purification of T-cell subsets and suppression assay

Both blood and duodenal biopsy specimens were obtained from the same patient with potential CD. Peripheral blood mononuclear cells were isolated from heparinized blood samples by density-gradient centrifugation (Ficoll, MP Biomedicals, LLC, Solon, OH). Freshly isolated mucosal cells were processed from intestinal mucosal explants after collagenase digestion. CD4+CD25+ and CD4+CD25− T cells were separated using the Dynabeads Regulatory CD4+CD25+ T-cell kit (Dynal-Biothec, AS, Oslo, Norway), as previously described (16). All purification steps were performed according to the manufacturer's instructions and collected cells were found to be >95% pure by flow cytometry. For suppression experiments, peripheral CD4+CD25− responder T cells (Tresp) and intestinal CD4+CD25+ (Treg) were cultured, respectively, at different ratios (1:1, 1:0.5, 1:0.25, 1:0.125, and 1:0) in U-bottom 96-well plates with RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum. Cells were stimulated for 3 days in the presence of Treg suppression Inspector (Miltenyi Biotec, Bergisch Gladbach, Germany) (16). Furthermore, the co-cultured Tresp/Treg cells were stimulated with 10 ng/ml of IL-15 (R&D System, Minneapolis, MN) or with 10 μg/ml of anti IL-10 receptor antibody (BD Pharmingen, 556011 clone 3F9). Proliferation of Tresp and Treg cells was determined according to our previous description (16).

At the end of the cell culture, supernatants were collected and analyzed for the content of IFNγ by enzyme-linked immunosorbent assay as previously described (16).

Statistical analysis

The non-parametric Mann–Whitney *U*-test and Student's two-tailed *t*-test were used for comparisons between the groups. The Pearson's correlation test was applied to analyze correlations between different parameters. A P value <0.05 was considered statistically significant. GraphPad Prism (GraphPad Software version 4.03, San Diego, CA) was used for statistical analysis and graphic representation.

RESULTS

Pro-inflammatory cytokines in duodenal mucosa from potential CD patients

To analyze the state of activation of mucosa T cells in early CD lesion, we examined RNA expression for IL-2, cytokines produced by activated Th cells following antigen stimulation, with a crucial role in proliferation and differentiation of CD4+ T cells, by real-time quantitative PCR in duodenal biopsies of CD patients. IL-2 RNA expression was found markedly increased in potential CD mucosa if compared with controls ($P<0.05$) and with active CD patients ($P<0.01$), in particular in Marsh 0 lesion ($P<0.05$). Surprisingly, we did not observe any difference between active CD and control patients (see **Supplementary Figure 1** online).

As the CD lesion is associated with a preferential Th1 response, to understand if in early state of CD there is already a differentiation of CD4+ T cells into Th1 effector subsets, we detected RNA expression for IFNγ, T-bet, and IRF-1, respectively, cytokine and transcription factors involved in Th1 polarization, by real-time quantitative PCR in CD patients. In potential CD mucosa, a clear

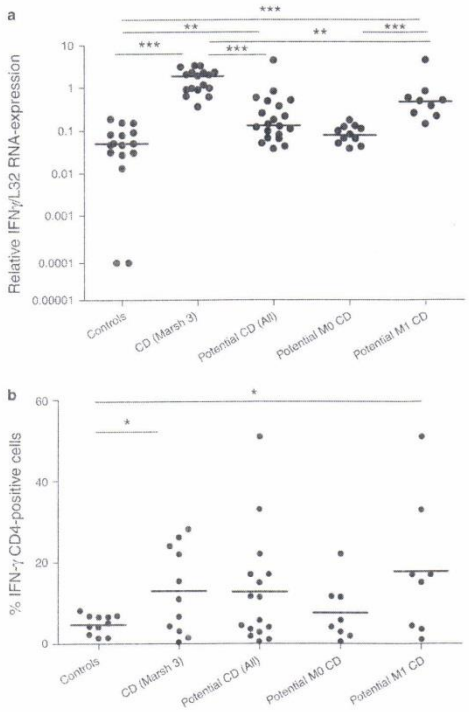

Figure 1. Increased expression of interferon γ (IFNγ) in duodenal mucosa of potential celiac disease (CD) patients with Marsh 1 lesion. (**a**) IFNγ-RNA expression measured by real-time quantitative-PCR in whole duodenal biopsies from patients with active CD (CD Marsh 3), potential CD including all subjects (All), or divided in potential CD with Marsh 0 (M0) and Marsh 1 (M1) mucosal lesion, and controls. Each point represents the relative amounts (relative IFNγ/L32 RNA expression) of IFNγ-RNA expression taken from a single subject. Horizontal bars indicate median values. $^{***}P<0.001$; $^{**}P<0.01$. (**b**) The percentage of IFNγ^{+}CD4^{+} lymphocytes in freshly isolated cells from mucosal biopsies of active CD (CD Marsh 3), potential CD and controls assessed by flow cytometry. Horizontal bars indicate mean values. $^{*}P<0.05$.

Th1 polarization was suggested by a significant increase of IFNγ-RNA expression if compared with control mucosa ($P<0.01$; **Figure 1a**). Interestingly, in potential CD mucosa, the RNA expression for IFNγ correlated significantly with IEL infiltration ($P<0.01$, Pearson $r=0.64$; data not shown). In fact, we found that in potential CD patients with Marsh 1 lesion, IFNγ-RNA expression was significantly overexpressed if compared with potential CD patients with Marsh 0 lesion and controls ($P<0.001$; **Figure 1a**). In Marsh 0, instead, IFNγ levels of RNA expression did not differ significantly from control patients. Remarkably, even if increased in potential CD mucosa, IFNγ levels were still significantly lower when compared with active CD ($P<0.001$; **Figure 1a**). Consistent with this finding, flow cytometry analysis showed a significantly higher percentage of CD4+IFNγ+ cells in active and in potential CD mucosa with Marsh 1 lesion than in potential CD with Marsh 0 lesion and in controls ($P<0.05$; **Figure 1b**).

T-bet-RNA expression was slightly enhanced in potential CD, but did not differ statistically from control or active CD mucosa (see **Supplementary Figure 2a**), most likely due to the wide confidence intervals. In active CD mucosa, it was significantly higher only when compared with control mucosa ($P<0.01$; see **Supplementary Figure 2a**).

IRF-1-RNA expression was similar in potential CD mucosa when compared with controls, but significantly lower if compared with active CD ($P<0.05$). We observed a significant increase of IRF-1-RNA expression in active CD if compared with control patients ($P<0.01$; see **Supplementary Figure 2b**).

As there are more and more evidence that the innate and adaptive immune stimuli collaborate to induce a pro-inflammatory Th1 response in CD (10), we investigated the expression of IL-15, a inflammatory cytokine involved in the innate response, in duodenal CD mucosa by immunohistochemistry. We chose immunohistochemical analyses instead of real-time quantitative PCR, as the RNA expression for IL-15 does not correlate with protein production because of a post-transcriptional regulation (21). We showed that potential CD patients had higher expression of IL-15 in the epithelium, as well as in the *lamina propria* if compared with controls ($P<0.05$), but lower than in active CD patients ($P<0.001$; **Figure 2**). In particular, IL-15 was increased in the early state of CD, in fact only patients with Marsh 0 lesion showed significantly higher levels of IL-15 than Marsh 1 patients and controls, both in the epithelium and *lamina propria* ($P<0.05$), although lower than in active CD patients ($P<0.01$; **Figure 2**).

IL-10/IFNγ ratio is increased in duodenal mucosa from potential Marsh 0 CD patients

As our data suggest that in potential CD, despite the absence of mucosal damage, pro-inflammatory cells seem to be activated and differentiating toward a Th1 pattern, we investigated whether this early Th1 response might be in some extent counter balanced by the presence of immunoregulatory cytokines. To this purpose, we analyzed RNA expression for IL-10, a key cytokine involved in immune regulation, by real-time quantitative PCR in duodenal mucosa of CD patients. In contrast to IFNγ, we observed a significant increase of IL-10-RNA expression in potential CD patients with Marsh 0 mucosal lesion if compared with those with Marsh 1 lesion ($P<0.001$), or with control patients ($P<0.001$; **Figure 3a**). Intracellular analysis of IL-10 by flow cytometry was next done on isolated cells from the CD mucosa. Interestingly, IL-10 was mainly produced by CD4+ cells, and consistently to RNA expression, the highest number of IL-10+CD4+ cells was observed in Marsh 0 potential CD patients. Although two patients with active CD had high number of IL-10-producing CD4+ cells, no differences were observed between active and control patient groups.

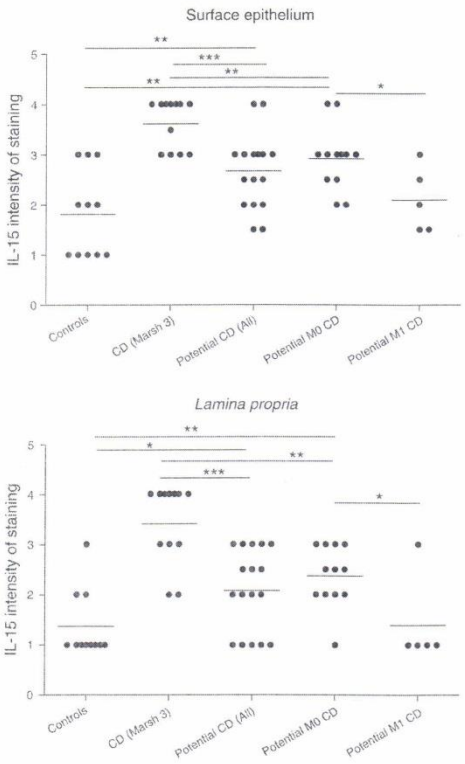

Figure 2. Interleukin-15 (IL-15) is increased both in surface epithelium and *lamina propria* of duodenal mucosa of active and potential celiac disease (CD) with Marsh 0 lesion. IL-15 expression in intestinal surface epithelium and *lamina propria* was evaluated in terms of staining intensity and graded on an arbitrary scale of staining from 1 to 4. The criteria for epithelium staining were as follows: 1=no signal, 2=weak signal present at the tip of villi with a patchy distribution, 3=strong signal located at the apex and along walls of the villi, and 4=very strong homogeneous signal. IL-15 staining within *lamina propria* mononuclear cells, was scored on a scale from 1 (no signal) to 4 (very strong signal) based on density and intensity of staining of positive cells. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

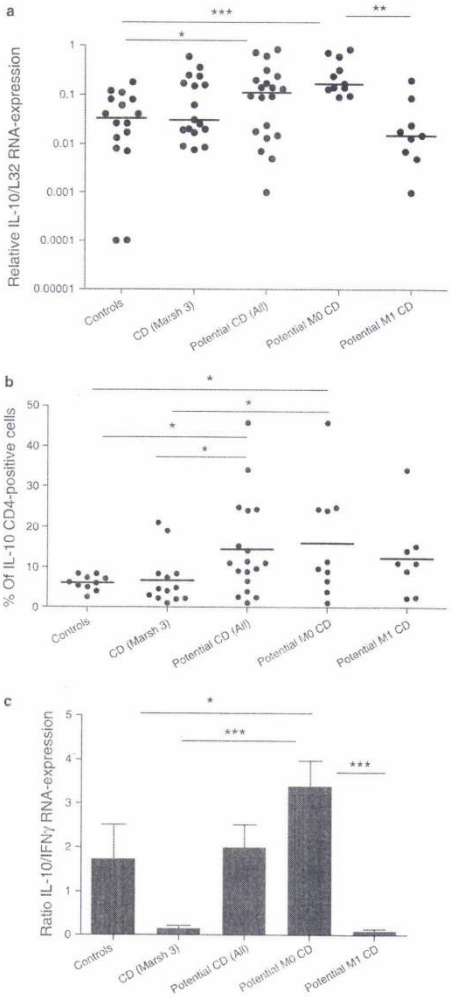

Figure 3. Interleukin-10 (IL-10) is increased in duodenal mucosa of potential celiac disease (CD) with Marsh 0 lesion. (**a**) IL-10 RNA expressions measured by real-time quantitative-PCR in whole duodenal biopsies from patients with active CD, potential CD including all subjects (All), or divided in potential CD with Marsh 0 (M0) and Marsh 1(M1) mucosal lesion, and controls. Each point represents the relative amounts (relative IL-10/L32 RNA expression) of IL-10 RNA expression taken from a single subject. Horizontal bars indicate median values. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. **(b)** Density of IL-10+CD4+ lymphocytes in freshly isolated cells from mucosal tissue assessed by intracellular IL-10 staining by flow cytometry. Each point represents the percentage of IL-10/CD4 double-positive cells gated in the region of mononuclear cells. Horizontal bars indicate mean values. $^{*}P<0.05$. **(c)** Relative ratio between IL-10 and IFNγ-RNA expression measured in whole duodenal biopsies. $^{*}P<0.05$; $^{***}P<0.001$.

Similarly, the percentage of IL-10+CD4+ cells in Marsh 1 potential CD patients, although increased, did not significantly differed from control or active CD (**Figure 3b**). When we compared the RNA expression of both IL-10 and IFNγ, an inverse correlation occurred and became significant in active CD patients (Pearson $r=0,62$, $P<0.01$; data not shown). As a consequence, the ratio between IL-10 and IFNγ-RNA expression was strongly reduced in active CD patients, as expected because of the high IFNγ levels. Remarkably, in Marsh 0 potential CD patients the ratio was

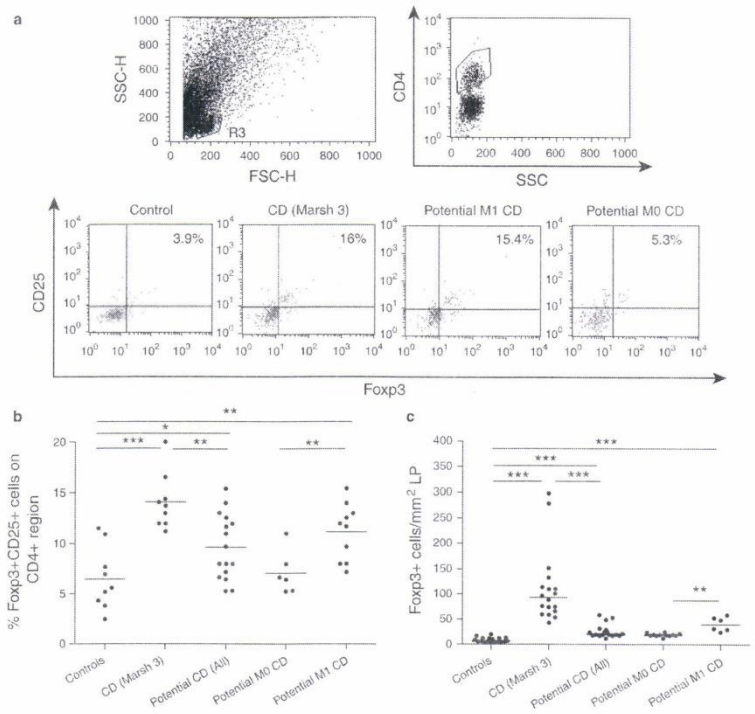

Figure 4. Foxp3+CD4+CD25+ cells are increased in duodenal mucosa of potential celiac disease (CD) with Marsh 1 lesion. **(a, b)** Intestinal lymphocytes were isolated from duodenal biopsies of patients with active CD (CD Marsh 3), potential CD including all subjects (All), or grouped in potential CD with Marsh 0 (M0) and Marsh 1 (M1) mucosal lesion, and controls; the percentage of Foxp3+CD4+CD25+ cells was analyzed by flow cytometry. **(a)** Representative dot plot from one experiment from each studied group is shown. Upper panel, lymphocytes gates on forward- and side-scatter properties to exclude dead and/or granular cells and representative gates of CD4 populations are shown. Below panel, number in each dot plot indicates the percentage of CD25+Foxp3+ cells on gated CD4+ cells. **(b)** Each point represents the percentages of the intestinal whole Foxp3+CD25+ T cells in total CD4+ cells in each subject. Horizontal bars indicate mean values. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. **(c)** Densities of Foxp3-expressing cells in *lamina propria* of CD patients and control subjects detected by immunohistochemistry. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

significantly enhanced when compared with potential CD patients with Marsh 1 lesion ($P<0.001$), with active CD ($P<0.001$), and even with control patients ($P<0.05$; **Figure 3c**).

Increased expression of Foxp3+Tregs in duodenal mucosa from potential CD patients

As regulatory T cells expressing Foxp3 are the main population involved in containing the activity of Th1 (22), we analyzed the percentage of Foxp3+CD25+ cells in intestinal CD4+ cell subset of CD patients by flow cytometry (**Figure 4a**). The percentage of Foxp3+CD25+CD4+ cells was significantly higher in potential CD compared with controls ($P<0.05$), although it was lower than in active CD ($P<0.01$; **Figure 4b**). In particular, CD patients with Marsh 1 lesion showed a percentage significantly higher than Marsh 0 and control patients ($P<0.01$; **Figure 4b**). Overall, we confirmed our previous observation (16) showing that the percentage of Foxp3+CD25+CD4+ cells was significantly higher in CD patients compared with controls ($P<0.001$; **Figure 4b**).

Immunohistochemical study confirmed the findings obtained by flow cytometry analysis. More specifically, in agreement with previous data (15,16,18), CD patients in the active phase of disease showed higher number of Foxp3+ cells in *lamina propria* than control and potential CD patients ($P<0.001$; **Figure 4c**). Furthermore, potential CD patients showed increased number of Foxp3-expressing cells in *lamina propria* compared with controls ($P<0.001$). In particular, potential CD patients with Marsh 1 lesion showed higher number of Foxp3+ cells when compared with those with Marsh 0 lesion ($P<0.01$), and with controls ($P<0.001$; **Figure 4c**).

Intestinal CD4+CD25+ cells in potential CD are regulatory T cells and their suppressive function is not impaired by IL-15

We have previously reported that Foxp3+CD25+CD4+ cells infiltrating celiac intestinal mucosa have a regulatory function (16). Here we investigated whether Foxp3+CD4+CD25+ cells isolated from potential CD mucosa had suppressive function, as well from atrophic mucosa. Intestinal CD4+CD25+ T cells from potential CD patients showed a hypoproliferative response, whereas Tresp cells proliferated vigorously, when they were cultured in the presence of a polyclonal stimulus (**Figure 5c**). In co-cultured, intestinal CD4+CD25+ T cells significantly suppressed the proliferation of Tresp cells both in terms of proliferation ($P<0.05$; **Figure 5a**) and IFNγ secretion ($P<0.05$, **Figure 5b**), and in cell dose-dependent way (**Figure 5c**). Furthermore, as recent studies in some animal models have suggested a role for IL-10 in the suppression mediated by Foxp3+Treg, we investigated the suppressive action of intestinal CD4+CD25+ T cells on proliferation of Tresp in the presence of anti-IL-10 receptor-alpha antibody. The IL-10 receptor blockade by specific antibody tends to reduce the ability of intestinal CD4+CD25+ T cells to suppress the proliferation of Tresp in an almost significant, in three co-cultured experiments ($P=0.08$; see **Supplementary Figure 3**).

As IL-15 is significantly increased in the small intestinal mucosa of potential CD and we have previously demonstrated that IL-15 alters the suppressive activity of intestinal CD4+CD25+ T cells in atrophic mucosa (16), we investigated the role of this cytokine in the early stage of CD. Unlike from active celiac patients, we demonstrated that in potential celiac patients IL-15 was not effective in counteracting intestinal Treg cell-mediated suppression of anti-CD3-activated Tresp cell in terms of proliferation and IFNγ production (**Figures 5a** and **b**). Moreover, a direct comparison of peripheral blood Treg cells from active CD and potential CD patients revealed that the surface density of IL-15 receptor-α was significantly lower in Treg cells from potential CD patients compared with active CD ($P<0.05$; **Supplementary Figures 4a** and **b**).

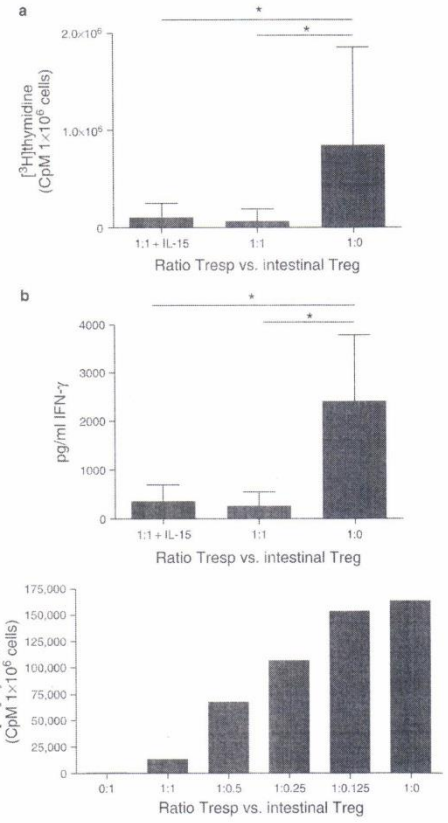

Figure 5. CD4+CD25+ Tregs from potential celiac disease (CD) mucosa are efficient in their suppressive activity and this is not reversed by interleukin-15 (IL-15). In co-culture, both proliferation (**a**) and IFNγ secretion (**b**) of peripheral responder CD4+ T cells (Tresp) of potential CD patients are efficiently inhibited by autologous intestinal CD4+CD25+ Treg. Proliferative responses were measured by [^{3}H]thymidine uptake. Interferon γ (IFNγ) secretion was evaluated in supernatants of co-cultures by an enzyme-linked immunosorbent assay test. Results from at least three potential patients are shown. *$P<0.05$ comparing the 1:0 vs. 1:1 ratio. (**c**) Treg cells efficiently suppressed Tresp cells proliferation in a cell dose-dependent manner (results are representative of one experiment). The effect of IL-15 on Treg function was evaluated co-culturing Tresp and Treg cells at 1:1 ratio in the presence of IL-15 (10 ng/ml), and both IFNγ production (**b**) and proliferation (**a**) were evaluated. Results from at least three potential patients are shown.

DISCUSSION

In this study, we provide evidence that in potential CD, despite the absence of mucosal damage, there are clear signs of inflammation; in particular, our data suggest that in duodenal mucosa of these patients, T cells seem to be activated and differentiating toward a Th1 pattern. Indeed, IL-2 RNA expression is increased in the mucosa of potential CD when compared with controls and active celiacs, confirming our previous data (23). Also, IFNγ-RNA expression, as well as the percentage of IFNγ+CD4+ cells, is significantly increased. Interestingly, IFNγ levels correlate with IEL infiltration, in agreement with previous data showing that the majority of IFNγ is produced in the epithelium (14). Similar to our findings, Westerholm-Ormio *et al.* (17) showed by *in situ* hybridization the higher density of IFNγ- and IL-2-positive cells in *lamina propria* of family members of patients with CD and dermatitis herpetiformis (17).

Confirming a trend to Th1 polarization, in our potential CD patients T-bet is more expressed than in controls, although not significantly. T-bet is a crucial transcription factor involved in Th1 differentiation (24). In particular, T-bet has a key role in the early phase of Th1 differentiation, as shown in a model of uveitis in rat (25). We hypothesize that also in CD the differentiation in Th1 starts in a early phase of disease, in fact in our potential CD with Marsh 0 lesion, the expression of T-bet-RNA tends to be higher, but most likely some of the signals required to properly sustain its

expression are lacking. IL-21 could be a candidate factor for this role. Recently, Fina *et al.* (26) have shown that IL-21 is enhanced in duodenal mucosa of CD, and interestingly, neutralization of IL-21 activity in *ex vivo* organ cultures of CD biopsies reduces both T-bet expression and IFNγ production (26). Interestingly, we have recently shown that potential CD has a marked reduced expression of IL-21-RNA when compared with CD and control patients (23).

In this same direction are our data that show the transcription factors downstream the signaling pathway elicited by IFNγ, are not activated in potential celiac mucosa, in fact in our potential patients IRF-1 is significantly lower if compared with active CD. IFNγ/IRF-1 signaling pathway has a key role in maintaining and expanding the local Th1 inflammatory response in CD disease (27–29). Our data suggest that this positive feedback loop is not activated in potential CD patients. So, we found sign of initial Th1 polarization but lack of the final mechanisms that amplify and stabilize the committed Th1 cell phenotype in the duodenal mucosa of CD at early stage. These data, and our recent observations (unpublished) showing the possibility to grow gliadin-specific T-cell lines from the duodenal mucosa of potential CD, suggest the presence of adaptive immune response responsible of the serum auto-antibodies production in potential CD.

Although CD is clearly associated with an involvement of the adaptive branch of the immune system, evidence indicates that a more complex pathogenic pattern controls the disease evolution. Recent studies suggest that the innate and adaptive immune stimuli synergize in inducing an inflammatory response that leads to massive expansion of IELs, and cytolytic attack of the epithelium (10). In this contest, IL-15 has an important role (11,12,21). This study continues in this direction and confirms the increase of IL-15 in active CD. In addition, we detect a significantly increased number of IL-15+ cells both in epithelium and *lamina propria* in potential CD, and particularly in those with Marsh 0 lesion, although in lower extend compared with active CD.

Potential CD patients have most of the mucosal inflammatory features of the gluten-induced immune-response, but they lack, for some time or even forever, the final destructive phase of it. We hypothesize that regulatory mechanisms might contribute to prevent the progression toward a complete mucosal damage. It is clear that IL-10 has a key role in limiting inflammatory responses *in vivo*, particularly in the intestine; mutations of IL-10 or IL-10 receptor are associated with inflammatory bowel disease, both in humans and in animal model (30–32). Somewhat surprisingly, in CD the levels of IL-10 mRNA are higher in untreated CD mucosa in comparison with treated CD patients and controls, however, the ratio IL-10/IFNγ transcripts is markedly reduced (13). In this study, we show that potential CD with Marsh 0 lesion has significantly higher levels of IL-10 RNA expression than Marsh 1 group and controls, whereas there are no differences with active CD; these data are in agreement with flow cytometry analysis, which demonstrates a significant increase percentage of IL-10+CD4+ lymphocytes producing cells isolated from potential CD patients with Marsh 0 lesion. The specific finding of this study is the inverse correlation between IL-10 and IFNγ-RNA expression, so the ratio between IL-10/IFNγ is increased in potential CD with Marsh 0 compared with active CD patients, potential CD Marsh 1 and controls, whereas in active CD and in potential CD Marsh 1 this ratio is significantly lower in comparison with controls. This suggests that in the early phase of the CD (Marsh 0) high levels of IL-10, which reflect a compensatory anti-inflammatory pathway, downregulate IFNγ production by counteracting also pro-inflammatory stimuli, such as IL-15; subsequently, during the infiltrative phase (Marsh 1) and then in active disease, for reasons that are not known, this same pathway is not sufficient to suppress the overwhelming Th1-mediated response (13).

In agreement with these and with previous data (18), we found, by both immunohistochemistry and flow cytometry *ex vivo* analysis, higher number of Foxp3+Tregs in potential CD with Marsh 1 lesion compared with Marsh 0 group. Thus, the increased density of Foxp3+Tregs seems to be correlated with the histological lesion suggesting that the immune system is actively trying to downregulate ongoing inflammation through the rapid redistribution of Foxp3+Tregs from the circulation or through their local proliferation. In line with our recent data obtained in patients with active CD (16), we investigated the suppressive capacity of CD4+CD25+ cells isolated from biopsies of potential CD in an *in vitro* co-culture assays. Our data show that intestinal CD4+CD25+ T cells of potential CD patients are able to exert their regulatory effects *in vitro* in terms of inhibition of proliferation and IFNγ secretion of Tresp cells. The exact mechanism of suppression by Foxp3+Tregs remains uncertain. *In vitro* studies have shown that the suppressive function is cell contact dependent and independent from cytokines (33,34). However, others studies in some animal models have suggested a role for IL-10 in the suppression by Foxp3+Treg (35,36). More recently, Huber *et al.* (37) have demonstrated that Foxp3+Tregs are able to control Th17 and Th17/Th1 cells in an IL-10-dependent manner *in vivo*. In our potential CD patients, we cannot exclude a possible role of IL-10; in fact, in our preliminary experiments the administration of anti-IL-10R receptor monoclonal antibody abrogates the ability of intestinal Treg to suppress the proliferation of Tresp in an *in vitro* co-culture assays.

In view of our recent data that show intestinal Tregs can be impaired in their suppression capacity by IL-15 in active CD (16), we investigated whether IL-15 might interfere with the suppressive activity of intestinal Treg cells also in potential CD. Interestingly, IL-15 is not able to impair the functions of suppression of Treg cells on Tresp cell in terms of proliferation and IFNγ production. The lower sensitivity to IL-15 of potential CD patients is likely to be due their reduced expression of IL-15 receptor.

In conclusion, our data suggest that regulatory mechanisms have a crucial role to downregulate the inflammation in early phase of CD. We have shown that in this condition there are two factors that maintain the suppressive action of Foxp3+Treg: they are the high IL-10 expression and a low sensitivity to pro-inflammatory stimuli, such as IL-15. Recent observations in animal model (38) have shown that Foxp3+Tregs may lose suppressive function and acquire diverse effector functions in the absence of IL-10 and the presence of an inflammatory state in the host. It remains to identify those conditions that might eventually overcome this immunoregulatory circuit favoring the expression of mucosal

damage. Potential CD proves to be a unique model to study the progression of CD, to identify key factors in the pathogenesis, and to offer useful information for prevention and alternative therapy to the gluten free diet.

CONFLICT OF INTEREST

Guarantors of the article: Riccardo Troncone, MD and Melissa Borrelli, PhD.

Specific author contributions: Melissa Borrelli was involved in study design, data analysis, supervised real-time quantitative PCR analyses, and drafting of the article. Virginia M. Salvati contributed to study design and real-time quantitative PCR analyses. Marianotonia Maglio contributed to study design, data analysis, and supervised immunohistochemical analyses. Delia Zanzi contributed to study design and performed flow cytometric analyses. Katia Ferrara and Sara Santagata performed purification of T-cell subsets, suppression assay, the enzyme-linked immunosorbent assay test, organ culture, and flow cytometric analyses. Domenico Ponticelli and Rosita Aitoro performed immunohistochemical analyses. Giuseppe Mazzarella supervised immunohistochemical analyses. Giuliana Lania contributed to real-time quantitative PCR analyses. Carmen Gianfrani contributed to data analysis and critical revision. Renata Auricchio and Riccardo Troncone were involved in patients' recruitment, study design, data analysis, drafting of the article, and critical revision.

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Potential competing interests: None.

Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Potential celiac disease (CD) relates to subjects with a normal small intestinal mucosa who are at increased risk of developing CD, as indicated by positive CD-associated serology.
- ✓ Sign of inflammatory reactions are present in intestinal mucosa of subjects with potential CD, as shown by the high level of IFNγ mRNA and densities of tumor necrosis factor-α-positive cells in the *lamina propria*.
- ✓ The number of FoxP3-expressing cells is increased in potential CD mucosa.

WHAT IS NEW HERE

- ✓ In duodenal mucosa of potential CD patients, T cells are activated and differentiating toward a Th1 pattern.
- ✓ An inverse correlation occurred between IFNγ and IL-10 RNA expression. As a consequence, the ratio between IL-10 and IFN-γ-RNA levels is overexpressed in potential CD with no signs of immune inflammation (Marsh 0).
- ✓ In co-culture assay, intestinal CD4+CD25+ T cells from potential CD patients exert suppressive effects on T responder cells, and their activity is not impaired by IL-15. The lower sensitivity to IL-15 of potential CD patients is likely to be due their reduced expression of IL-15 receptor.

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4. Correlation between levels of anti-TG2 and mucosal damage degree

Coeliac disease (CD) is characterized by highly specific autoantibodies directed against transglutaminase 2 (TG2) (51). It is now well known that serum levels of anti-TG2 correlate with intestinal damage (81,82). This finding has been considered in the recently revised European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria, and used to avoid biopsies in symptomatic patients with high titres of anti-TG2 (83). CD-specific autoantibodies disappear from serum after the beginning of a gluten-free diet (GFD) (84).

Anti-TG2 antibodies are found both in blood and small intestine, where they are produced, and have been shown to co-localize with extracellular TG2 in the active phase of the disease (53). In recent decades several techniques have been used to reveal intestinal production of anti-TG2 antibodies, such as measurement in faeces (85) or duodenal juice (86), or in supernatants of cultured biopsies (59,60,62), the detection of mucosal deposits (53) or of plasma cells secreting them (87) and their expression by phage display library of RNA coding (52). Some assays were considered unreliable as diagnostic tests (88); others, even if with high diagnostic sensitivity and specificity, too demanding to be performed routinely (52).

We have demonstrated that the measurement of antibodies released into culture supernatants is more sensitive than detection of their deposits to assess intestinal production of anti-TG2 in patients with potential CD (89). Picarelli et al. have shown recently that the organ culture system is a useful tool to assist the histology in diagnosing CD, mainly in cases without villous atrophy or in seronegative patients (90). Concerning patients on a gluten-free diet (GFD), the same authors showed that anti-endomysium (EMA) was not found in supernatants of biopsy samples cultured with medium alone, but was detected when biopsy samples from the same patients were treated with gliadin

peptides (91). Furthermore, Maki et al. (92) showed that, in the organ culture system conducted with biopsies of treated CD patients, gliadin induced secretion of autoantibodies into culture supernatants, reduced epithelial cell height and increased the density of lamina propria CD25+ cells. However, these changes could be demonstrated only in biopsies from CD patients who had recently started a GFD, in when the small-intestinal mucosal TG2-specific IgA autoantibody deposits were still present.

It is well known that the disappearance of serum anti-TG2 is observed progressively after the beginning of a GFD. It has been considered a useful tool to evaluate the diet compliance, although it is not yet clear if it is helpful to assess complete histological recovery (93).

The aims of our study were to correlate, in coeliac patients, the titres of intestinal antibodies and the degree of mucosal damage and to investigate the effect of GFD on their intestinal production.

We showed that the intestinal production of these autoantibodies correlated with mucosal lesion degree only in active CD. Potential CD patients with Marsh 0 mucosa produced titres of intestinal anti-TG2 antibodies comparable to those with Marsh 1. Moreover, in this potential coeliac population intestinal anti-TG2 titres correlated with an increased number of CD25+ mononuclear cells in lamina propria, a proven marker of mucosa inflammation (94).

Studies on the follow-up of CD patients on GFD (95) have shown that titres of circulating anti-TG2 antibodies decreased significantly during the first 12 months of the diet and then disappeared in the next 12 months. The disappearance of serum CD-specific autoantibodies was correlated with GFD compliance, but not always associated with the total recovery of small intestinal mucosa (93). Our data appear to show that the early disappearance of

circulating specific CD autoantibodies, following a strict GFD, does not mean the end of their intestinal production. After the disappearance from serum, anti-TG2 antibodies as deposits would disappear slowly from the intestine, and only after a long period of GFD, small intestinal mucosa would cease to produce them.

Finally, our data show that in vitro 24-h PTG stimulation was not able to induce a statistically significant increase of antibody secretion in short-term GFD CD patients as well

as in patients on long-term GFD.

These data have been published as Article on *Clinical Experimental Immunology* , for the manuscript see below.

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Intestinal titres of anti-tissue transglutaminase 2 antibodies correlate positively with mucosal damage degree and inversely with gluten-free diet duration in coeliac disease

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Summary

It has always been known that anti-tissue transglutaminase 2 (anti-TG2) antibodies are produced in the small intestine. Their serum titres correlate with mucosal damage degree and decrease on a gluten-free diet (GFD).

We aimed to correlate intestinal anti-TG2 antibodies levels with degree of mucosal damage and GFD duration.

Thirty-four active, 71 potential and 24 CD patients on GFD for at least 2 years were enrolled. Anti-TG2 deposits were detected in intestinal biopsies by double immunofluorescence. Biopsies were cultured for 24 h with medium, and with gliadin peptic tryptic digest (PTG) or A-gliadin peptide 31–43 (P31-43). Anti-TG2 antibodies secreted into supernatants were measured by enzyme-linked immunosorbent assay (ELISA). All active CD patients secreted high titres of anti-TG2 antibodies into culture medium that increased with the worsening of mucosal injury (Spearman's $r = 0{\cdot}71$; $P < 0{\cdot}0001$). Seventy of 71 potential CD patients and 15 of 24 treated CD patients secreted low titres of anti-TG2 antibodies into supernatants, eight of nine negative treated patients being on GFD for more than 10 years. An inverse correlation between antibody titres and duration of GFD was found, (Spearman's $r = -0{\cdot}52$; $P < 0{\cdot}01$). All active, 53 of 71 potential and six of 24 treated, CD patients showed anti-TG2 mucosal deposits. Five of six positive treated CD patients had been on GFD for fewer than 6 years and were also positive for secreted anti-TG2. In treated patients, PTG/P31-43 was not able to induce secretion of anti-TG2 antibodies into culture medium.

Measurement of anti-TG2 antibodies in biopsy supernatants proved to be more sensitive than detection by immunofluorescence to reveal their intestinal production. Intestinal antiTG2 antibodies titres correlated positively with the degree of mucosal damage and inversely with the duration of GFD.

Keywords: anti-tissue transglutaminase 2, coeliac disease, gluten-free diet, intestinal antibodies

Introduction

Coeliac disease (CD) is a T cell-mediated inflammatory disorder of the small intestine caused by gluten in genetically susceptible individuals [1]. CD is characterized by highly specific autoantibodies directed against transglutaminase 2 (TG2) [2]. It is now well known that serum levels of anti-TG2 correlate with intestinal damage [3,4]. This finding has been considered in the recently revised European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) criteria, and used to avoid biopsies in symptomatic patients with high titres of anti-TG2 [5]. CD-specific autoantibodies disappear from serum after the beginning of a gluten-free diet (GFD) [6].

Anti-TG2 antibodies are found both in blood and small intestine, where they are produced, and have been shown to co-localize with extracellular TG2 in the active phase of the disease [7]. In recent decades several techniques have been used to reveal intestinal production of anti-TG2 antibodies, such as measurement in faeces [8] or duodenal juice [9], or

in supernatants of cultured biopsies [10–12], the detection of mucosal deposits [7] or of plasma cells secreting them [13] and their expression by phage display library of RNA coding [14]. Some assays were considered unreliable as diagnostic tests [15]; others, even if with high diagnostic sensitivity and specificity, too demanding to be performed routinely [14].

Recently we have demonstrated that the measurement of antibodies released into culture supernatants is more sensitive than detection of their deposits to assess intestinal production of anti-TG2 in patients with potential CD [16]. Picarelli *et al.* have shown recently that the organ culture system is a useful tool to assist the histology in diagnosing CD, mainly in cases without villous atrophy or in seronegative patients [17]. Concerning patients on a gluten-free diet (GFD), the same authors showed that anti-endomysium (EMA) was not found in supernatants of biopsy samples cultured with medium alone, but was detected when biopsy samples from the same patients were treated with gliadin peptides [18]. Furthermore, Maki *et al.* [19] showed that, in the organ culture system conducted with biopsies of treated CD patients, gliadin induced secretion of autoantibodies into culture supernatants, reduced epithelial cell height and increased the density of lamina propria $CD25^+$ cells. However, these changes could be demonstrated only in biopsies from CD patients who had recently started a GFD, in when the small-intestinal mucosal TG2-specific IgA autoantibody deposits were still present.

It is well known that the disappearance of serum anti-TG2 is observed progressively after the beginning of a GFD. It has been considered a useful tool to evaluate the diet compliance, although it is not yet clear if it is helpful to assess complete histological recovery [20].

The aims of our study were to correlate, in coeliac patients, the titres of intestinal antibodies and the degree of mucosal damage and to investigate the effect of GFD on their intestinal production.

Material and methods

Patients

One hundred and twenty-nine CD patients were enrolled at the Department of Medical Translational Sciences, Section of Pediatrics, University Federico II in Naples. One hundred and five patients (range 2–16 years, mean = 6 years) underwent a small intestinal biopsy because of clinical suspicion of CD. They had serum levels of anti-TG2 over the cut-off (7 U/ml) and/or positive EMA antibodies. Thirty-four of 105 patients showed villous atrophy with a grade Marsh 3 (Marsh 3a, $n = 13$; 3b, $n = 11$; 3c, $n = 10$) [21]; they received a diagnosis of CD. Seventy-one of 105 patients showed an architecturally normal intestinal mucosa with a grade of 0/1 (Marsh 0, $n = 34$; 1, $n = 37$); they were coded as potential CD patients. Twenty-four of 129 patients (range 8–48 years, mean = 19 years) on a GFD for at least 2 years also underwent a small intestinal biopsy. All patients on a GFD had architecturally normal intestinal mucosa (Marsh 0, $n = 10$; 1, $n = 14$) and serum levels of anti-TG2 below the cut-off. At the time of their initial diagnosis, four of 24 patients were potential CD and when they started the GFD presented a mucosa with Marsh 0 or 1 lesion; in fact, they were put on a GFD because of clinical symptoms that disappeared after beginning the GFD. Immunoglobulin (Ig)A deficiency was excluded in all patients.

Duodenal biopsy and organ culture system

During upper gastrointestinal endoscopy, at least five duodenal biopsies were taken from all patients. Two fragments were fixed in 10% formalin, paraffin-embedded and then treated for histological and morphometric analysis. Moreover, for potential CD patients, 4-μm-thick paraffin haematoxylin-stained sections were used to evaluate villous height crypt depth ratio (Vh/CrD); Vh/CrD ≥ 2 was considered normal [22]. One of the duodenal specimens was embedded in a cryostat-embedding medium (Killik; Bio-Optica, Milan, Italy) and stored in liquid nitrogen until used. The remaining fragments were cultured for 24 h at 37°C with medium alone. Moreover, fragments from CD patients on a GFD were cultured for 24 h either in the presence or absence of peptic–tryptic digest of gliadin (PTG, 1 mg/ml) or A-gliadin peptide P31-43 (100 μg/ml). Organ culture was performed as reported previously [23]. After 24 h of culture, the tissues were embedded in optimal cutting temperature (OCT) and stored in liquid nitrogen. The culture supernatants were collected and stored at −80°C until analysed.

Measurement of anti-TG2 IgA antibodies secreted into culture supernatants

Mucosal anti-TG2 IgA antibodies secreted into culture supernatants were measured in undiluted supernatants by enzyme-linked immunosorbent assay (ELISA EU-tTG IgA kit; Eurospital, Trieste, Italy), according to the manufacturer's instructions. When the value of anti-TG2 was higher than the last point of standard curve, supernatants were diluted 1 : 10 in culture medium. The cut-off value for anti-TG2 IgA antibodies in culture supernatants was 2·8 U/ml, as calculated previously in our laboratory [16].

Detection of intestinal deposits of anti-TG2 IgA antibodies and immunohistochemistry

The presence of intestinal deposits of anti-TG2 IgA was investigated in non-cultured fragments from all CD patients. Five-μm cryostat sections were stained using a double-immunofluorescence method, as described previously [24]. The stained sections were evaluated using a

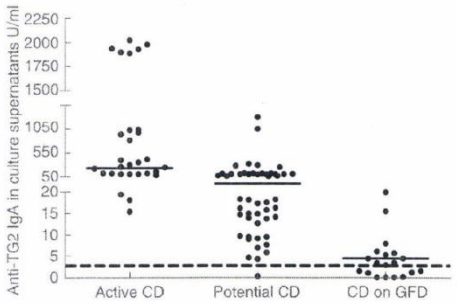

Fig. 1. Titres of anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A, expressed as U/ml, in medium culture supernatants. The dotted line represents the cut-off of 2·8 U/ml; horizontal lines represent median values. The *y*-axis is divided into three segments in the same graph to also show the low titres of anti-TG2 secreted into culture medium by potential and treated coeliac disease (CD) patients. GFD, gluten-free diet.

confocal microscope (LSM510; Zeiss, Oberkochen, Germany). Immunohistochemical stainings for $CD3^+$ and γδ-T cell receptor $(TCR)^+$ intraepithelial lymphocytes and $CD25^+$ lamina propria mononuclear cells, as well as evaluation of cell densities, were performed in potential CD patients, as reported previously [25].

Statistics

Statistical analysis was performed using GraphPad Prism 4 for Windows, version 4·03 (GraphPad Software, San Diego, CA, USA). Quantitative data were expressed as medians. The Mann–Whitney *U*-test was used to compare titres of anti-TG antibodies in supernatants between the groups. A paired *t*-test was used to compare changes of anti-TG2 titres in supernatants after culture with PTG/P3143 within the group of CD patients on a GFD. Spearman's correlation was used to compare anti-TG2 titres in supernatants and laboratory values as Vh/CrD ratios, $CD3^+$ intraepithelial lymphocyte (IEL) density, γδ-TCR^- IELs density and $CD25^+$ mononuclear cells in potential CD patients. Moreover, Spearman's correlation was used to compare anti-TG2 titres in supernatants and duration of the GFD in treated CD patients and to compare anti-TG2 secreted into culture medium and degree of villous atrophy (according to Marsh classification) in active CD patients. A *P*-value $< 0{\cdot}05$ was considered statistically significant.

Stepwise canonical discriminant analysis was adopted to select variables that discriminated between the three groups of cases. Wilks' lambda was used to estimate the capacity of each variable to discriminate among the three groups, ranging between 0 and 1, where 1 = complete overlap and 0 = complete separation. The stepwise multivariate procedure selects the first variable that minimizes Wilks' lambda, then includes the subsequent variables progressively, according to their contribution to lowering Wilks' lambda. The variance ratio *F* provides an estimate of each variable's contribution to the discrimination between groups.

Ethical approval

Written informed consent was obtained from adult patients and from parents of children enrolled. The study protocol was approved by the Ethical Committee of the University 'Federico II' Naples, Italy (CE 230/05).

Results

IgA anti-TG2 in culture supernatants

All CD patients in the active phase of disease secreted high titres of anti-TG2 antibodies into culture supernatants (range 15·3–2000 U/ml; median = 222·1 U/ml) (Fig. 1). The antibody titre increased gradually with the worsening of mucosal injury; that is, from grade Marsh 3a to 3c lesion (Fig. 2), with a Spearman's correlation coefficient = 0·71 ($P < 0{\cdot}0001$). All potential CD patients, except one, secreted anti-TG2 antibodies into culture supernatants. The antibody titres were variable, ranging from 4·2 to 1247·0 U/ml (median = 26·4 U/ml) (Fig. 1); however, there was no statistical difference between Marsh 0 and 1 lesions (Marsh 0 median = 29·9; 1 = 23·8; $P > 0{\cdot}05$) and between potential and Marsh 3a CD patients (Fig. 2). Moreover, titres of secreted anti-TG2 correlated with serum titres of anti-TG2 (Table 1), confirming our previous data [16]. In 62 potential CD patients an immunohistochemical analysis of intestinal biopsies was performed and the correlation between the antibody titres of anti-TG2 secreted into culture supernatants with other parameters such as villous height/crypt

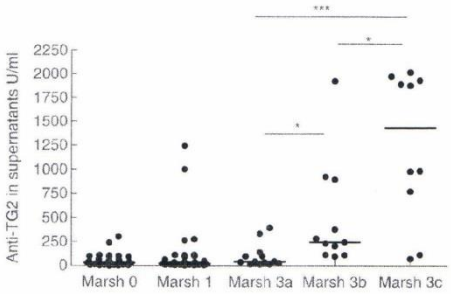

Fig. 2. Anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A, expressed as U/ml, secreted into culture medium by active (Marsh 3a, 3b, 3c) and potential (Marsh 0, 1) coeliac disease (CD) patients according to grade of mucosal lesion. $^*P < 0{\cdot}05$; $^{***}P < 0{\cdot}001$; Mann–Whitney *U*-test.

Table 1. Spearman's correlation between titres of anti-transglutaminase 2 (TG2) secreted into culture supernatants by potential coeliac disease (CD) patients and markers.

Marker	Spearman's *r* coefficient	*P*
Vh/CrD	−0·19	0·155
$CD3^+$ density	0·22	0·084
$\gamma\delta$-TCR^+ density	0·19	0·138
$CD25^+$ density	0·25	<0·05
Anti-TG2 in serum	0·34	<0·01

Vh/CrD, villous height crypt depth ratio; $CD3^+$, intraepithelial lymphocytes; $\gamma\delta$-TCR^+, intraepithelial lymphocytes express the gamma/delta T cell receptor; $CD25^+$, activated lamina propria mononuclear cells.

depth ratio, density of intraepithelial lymphocytes $CD3^+$, $\gamma\delta$-TCR^+ and the $CD25^+$ mononuclear cell density in lamina propria was evaluated. Our data showed that only lamina propria $CD25^+$ mononuclear cell density was correlated with increased levels of secreted anti-TG2 antibodies (Spearman's correlation coefficient = 0·25; $P < 0·05$) (Table 1).

When CD patients on a GFD were considered, 15 of 24 secreted low titres of anti-TG2 antibodies (range 3·3–27·2 U/ml; median = 4·35 U/ml) (Fig. 1). Antibody titres did not correlate with the mucosal lesion degree (Spearman's $r = 0·02$, $P = 0·9$). Eight of nine negative patients had been on a GFD for more than 10 years. The ninth negative patient had been on GFD for less than 2 years, but he had a Marsh 1 lesion at diagnosis. All four patients in remission who had Marsh 0/1 mucosa when put on a GFD (potential CD patients) produced lower titres of anti-TG2 compared to patients with similar duration of the GFD but with Marsh 3 mucosal lesion at the time of diagnosis. Only one patient on GFD for 12 years continued to produce low amounts of anti-TG2 antibodies (4·34 U/ml). He also had type 1 diabetes. There was an inverse correlation between antibody titres and duration of GFD (Spearman's correlation coefficient = −0·52, $P < 0·01$).

Finally, CD patients on a GFD did not show a significant increase in antibody titres after *in-vitro* challenge with PTG or P31-43. PTG/P31-43 stimulation was not able to induce an increase of anti-TG2 antibody secretion into culture supernatants of small intestinal fragments, even in the nine patients with no basal production of intestine anti-TG2 antibodies (data not shown).

Intestinal deposits of anti-TG2 IgA

Mucosal deposits of IgA anti-TG2 were detected in duodenal mucosa of all active and 53 of 71 potential CD patients. Among the 18 potential CD patients without mucosal deposits, only one was also negative for supernatant anti-TG2. Considering CD patients on a GFD, six of 24 showed mucosal deposits. Five of six had been on a GFD for less than 6 years and secreted anti-TG2 into culture medium, with titres ranging from 22 to 27 U/ml. The last patient (also affected by type 1 diabetes) had been on a GFD for 12 years. Patients on a GFD with mucosal deposits secreted anti-TG2 into culture supernatants with levels higher than the patients without them ($P < 0·01$, Fig. 3). Moreover, the intensity of mucosal deposit staining of IgA anti-TG2 was correlated directly with titres of secreted anti-TG2 (Spearman's $r = 0·63$, $P < 0·001$).

A concordance of 76·7% was observed between the two techniques used to evaluate the mucosal production of anti-TG2 antibodies in our study population.

Multivariate discriminant analysis

As described in the Methods, we attempted a multivariate discriminant analysis in order to identify those variables that, in the multivariate model, are the most efficient to discriminate among groups. As shown in Table 2, Wilks' lambda was lowered exclusively by the two variables related to the production of anti-TG2 in the mucosa (mucosal deposits and anti-TG2 production into culture supernatants). The anti-TG2 antibody level in the serum did not add any significant improvement to the discriminating function after inclusion of the above variables.

By using the discriminant equation obtained, it is possible to allocate each individual case to the group for which he has the highest probability computed by the two selected variables. When we compared active and potential cases,

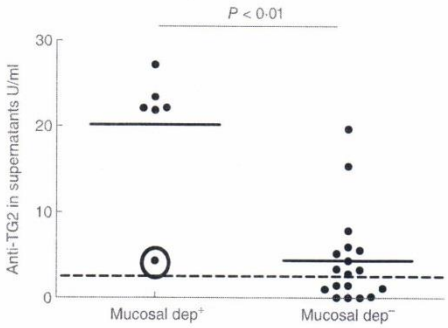

Fig. 3. Coeliac disease (CD) patients on gluten-free diet (GFD) with or without mucosal deposits coded as mucosal dep^+ and mucosal dep^-, respectively. Mucosal dep^+ patients secreted anti-transglutaminase 2 (TG2) immunoglobulin (Ig)A antibodies into culture medium with titres significantly higher than mucosal dep^- patients. The dotted line represents the cut-off of 2·8 U/ml. The circle shows the type 1 diabetes patient on GFD for 12 years who produced low amounts of anti-TG2 antibodies; Mann–Whitney *U*-test.

Table 2. Multivariate discriminant analysis.

Variables entered/removed[†,‡]				
		Wilks' lambda		
			Exact *F*	
Step	Entered	Statistic	Statistic	Sig.
1	Mucosal deposits	0·579	37·156	0·000
2	Anti-TG2 into culture medium	0·506	20·492	0·000

At each step, the variable that minimizes the overall Wilks' lambda is entered. [†]Minimum partial *F* to enter is 3·84. [‡]Maximum partial *F* to remove is 2·71. TG2, transglutaminase 2.

73·5% of active CD were identified correctly by the equation *versus* 95·8% of potential cases. The equation was obviously less efficient to discriminate potential from remissions (65 *versus* 79·2%).

Discussion

High titres of anti-TG2 antibodies are found in the serum of most coeliac patients. They are related to mucosal damage severity and disappear from the serum in patients on a GFD. Anti-TG2 antibodies are synthesized in the intestinal mucosa and, as described previously, several techniques have been devised to prove this. To identify the most suitable test for detection of the intestinal production of anti-TG2 antibodies, we have recently compared two of these techniques in potential CD patients: the search for anti-TG2 intestinal deposits and measurement of the same antibodies secreted into supernatants after organ culture [16]. Our data showed higher sensitivity and specificity of the anti-TG2 dosage into culture supernatant for this purpose [16]. This test is objective, not bound to operator experience, not too demanding and not expensive.

In this study, we confirmed previous results and showed for the first time that there was a correlation between levels of intestinal anti-TG2 antibodies and severity of mucosal damage. A similar correlation has already been found between serum anti-TG2 antibody levels and duodenal histology in paediatric [20,26] and adult [26,27] coeliac populations. However, our data showed that the intestinal production of these autoantibodies correlated with mucosal lesion degree only in active CD. Potential CD patients with Marsh 0 mucosa produced titres of intestinal anti-TG2 antibodies comparable to those with Marsh 1. Moreover, in this potential coeliac population intestinal anti-TG2 titres correlated with an increased number of $CD25^+$ mononuclear cells in lamina propria, a proven marker of mucosa inflammation [28].

Furthermore, we investigated intestinal production of anti-TG2 with the same two assays in CD patients on a GFD for at least 2 years. Two-thirds (63%) of patients on a GFD secreted low amounts of these autoantibodies, while mucosal deposits were detected in fewer than one-third (25%) of patients. This means that in most cases anti-TG2 antibodies, when produced, do not accumulate as mucosal deposits; in these cases the absence of mucosal deposits could depend upon the low titres and decreasing affinity of anti-TG2 antibodies for their antigen as a consequence of a long period of GFD. In our hands, anti-TG2 detection in culture medium seems to be more sensitive in revealing their intestinal production. Studies on the follow-up of CD patients on GFD [29–31] have shown that titres of circulating anti-TG2 antibodies decreased significantly during the first 12 months of the diet and then disappeared in the next 12 months. The disappearance of serum CD-specific autoantibodies was correlated with GFD compliance, but not always associated with the total recovery of small intestinal mucosa [20]. Our data appear to show that the early disappearance of circulating specific CD autoantibodies, following a strict GFD, does not mean the end of their intestinal production. After the disappearance from serum, anti-TG2 antibodies as deposits would disappear slowly from the intestine, and only after a long period of GFD, small intestinal mucosa would cease to produce them.

Anti-TG2 antibodies in coeliac mucosa are produced by TG2-specific plasma cells that reduce their number on GFD [13]. The density of these intestinal plasma cells in treated CD patients is lower than in active CD, but higher than in non-CD subjects. Recent evidence [13,32] shows that secreting plasma cells from human intestine can live *in vitro* for several weeks and could potentially live *in vivo* for months or years, thanks to a microenvironment favourable to their long-term survival. Only after a long period of GFD do these secreting cells disappear and, as result, intestinal anti-TG2 production ends.

Finally, our data show that *in vitro* 24-h PTG stimulation was not able to induce a statistically significant increase of antibody secretion in short-term GFD CD patients as well as in patients on long-term GFD. The ability of gliadin peptides to induce antibody secretion (EMA and/or anti-TG2 antibodies) into supernatants of cultured biopsies from treated CD patients has been investigated widely in the last 20 years, with contradictory results [14,17–19]. Maki *et al.* [19] hypothesized that secretion of specific CD autoantibodies from cultured small intestinal biopsies of treated CD patients is related to the presence of anti-TG2 mucosal deposits that are usually detected in those patients who have been on a GFD for a short time-period. Our data appear to show that secretion of anti-TG2 antibodies is detected regardless of the presence of mucosal deposits. Absence or a very low density of TG2-specific plasma cells and/or the short duration of organ culture might explain the inability of gliadin peptides to stimulate antibody secretion in treated CD patients.

In conclusion, our results show that the measurement of intestinal antibodies in biopsy supernatants represents a valid, quantitative test to investigate the production of these autoantibodies at each stage of disease. In our hands, it is

more sensitive than detection of mucosal deposits by immunofluorescence. The production of intestinal anti-TG2 antibodies could represent the very early stage of gluten-induced mucosal injury, when the integrity of small intestinal mucosa is still conserved and anti-TG2 are not detectable in serum. This test has a potentially great impact in clinical practice to unravel the wide spectrum of gluten sensitivity.

Disclosure

The authors declare no conflicts of interest.

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CHAPTER II

"Food Allergy"

1. Diagnosing and Treating Food Allergy

Food allergy (FA) is a major health issue in western societies affecting between 5 and 10 % of young children (96). During the last decade in the United States pediatric FA diagnosis rates increased by 18%, however it has not been determined whether these findings are related to increased awareness and reporting. In any case, the use of specific medical diagnostic codes for FA does represent a real increase of the disease (97). It has been estimated that FA, in the United States alone, accounts for 30,000 emergency room visits and 150 deaths per year (98). In Italy, the number of hospital admissions due to food-induced anaphylaxis doubled in the last 5 years (99).

Food allergies can be broadly divided into IgE (type I hypersensitivity)- and non-IgE (usually type IV hypersensitivity)-mediated diseases; or mixed, involving other immunoglobulins, immune complexes and/or cell-mediated mechanisms. These differ in clinical presentation, diagnostic testing, and prognosis (100). IgE-mediated reactions are characterized by an acute onset of symptoms generally within 2 h after ingestion of or exposure to food. IgE-mediated reactions to food typically involve the skin, gastrointestinal tract, and respiratory tract and may include systemic reactions (anaphylactic shock). Non-IgE-mediated immunological reactions (e.g., cell-mediated) include food–protein-induced enterocolitis, proctocolitis, and enteropathy syndromes. These conditions primarily affect infants or young children who present with abdominal complaints, such as vomiting, abdominal cramps, diarrhea, and occasionally blood in the stool and failure to thrive or poor weight gain. Examples of FA co-morbidities with mixed IgE- and non-IgE-mediated causes include eosinophilic gastrointestinal diseases and atopic dermatitis (101).

Oral food challenge still represents the gold standard for the diagnosis of FA in order to avoid unjustified diets. When the food considered for the challenge is still part of the patient's diet, a strict elimination diet should be prescribed for at least 2 weeks before the OFC. The OFC is done by feeding gradually increasing amounts of the suspected food under observation by a physician over a period of hours, protracted for days when no immediate reaction occurs.

Because the procedure carries a small risk of anaphylaxis, it should be conducted in a supervised medical setting where resuscitation equipment is available. The main problems of OFC are related to the wide variety of symptoms possibly related to immunological mechanism of FA that lead to difficulties in the interpretation of results and to the optimal timing and dosage of this procedure. A rather complex, double-blind placebo- controlled food challenge (DBPCFC), routinely used in research, is recommended in clinical settings only when patients report entirely subjective symptoms; whereas an open OFC without placebo is commonly used in children under the age of 3 years and when objective symptoms are present (102-104). Unfortunately, the diagnosis of FA is frequently incomplete, incorrect, or self-reported, and a correct diagnostic work-up, confirmed by OFC, seems to be adopted in only a minority of cases (105).

Although numerous therapeutic treatment options are currently being investigated, dietary avoidance remains the primary treatment for FA (106). Inadequate nutritional status, growth, and dietary intakes have been demonstrated in children with FA (107). Altered growth status may be due to potential loss of nutrients caused by continued allergic inflammation and/or abnormal intestinal permeability caused by noncompliance with the diet, unbalanced diet and additional undiagnosed FA or inappropriate substitute foods (107). This highlights the need to make every effort to optimize nutrition because inadequate nutrient intake may worsen the risk of lower growth rates in this population. The elimination diet should be

considered carefully depending on: mechanism of FA; symptoms; nutritional status; and concomitant factors like food aversive behavior. A properly managed, well-balanced elimination diet prescribed with the help of certified dietitians, can lead to resolution of symptoms while maintaining or even optimizing nutritional status.

Recent data strongly suggest that gut microbiota is important for oral tolerance development (108). Administration of the probiotic Lactobacillus rhamnosus GG (LGG) to food-allergic children (age\2 years, challenge-proven and affected by mild-to-moderate eczema) improved the eczema score significantly (108). Studies in infants with eczema who received formulas supplemented with LGG showed benefits in decreasing gastrointestinal symptoms (109). For instance, after a challenge study in infants allergic to cow's milk proteins, fecal IgA levels were detected to be higher, and TNF-a levels were lower in the LGG applied group compared to the placebo (108). Nermes et al. (110) showed a significant decrease in IgA- and IgM-secreting cells in infants with atopic dermatitis treated with extensively hydrolyzed casein formula (eHCF) supplemented with LGG, suggesting that this particular probiotic is able to enhance gut barrier function and accelerate immunological maturation in infants with FA.

Correct diagnosis of FA is crucial to ensure appropriate patient care. The essential criterion is a clear response to elimination diet, and other diagnostic tests are secondary to this. OFC plays a crucial role in the diagnostic approach to a child with suspected FA, but it is largely underutilized.

Potential responsible factors contributing to the lack of a correct diagnostic work-up in the vast majority of cases could be numerous, and include lack of training on the procedure, increased reliability on screening methods, extensive time needed, fear of risk, and suboptimal fee reimbursement. A comprehensive nutrition assessment with appropriate intervention is warranted in all children with FAs to meet nutrient needs and optimize

growth. Frequently an elimination diet is absolutely necessary to prevent potentially life-threatening food allergic reactions.

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GASTROENTEROLOGY (S. GUANDALINI, SECTION EDITOR)

Diagnosing and Treating Food Allergy

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Abstract Food allergy (FA) is defined as an abnormal immunological reaction to food proteins. Over 90 % of FAs in childhood are caused by eight foods: cow's milk, hen's egg, soy, peanuts, tree nuts, wheat, fish and shellfish. The diagnostic work up for a child with suspected FA includes detailed medical history, physical examination, FA screening tests and response to elimination diet and to oral food challenge. Sometimes additional diagnostic tools to explore intestinal damage and function could be adopted. Currently, the only treatment for FA relies on strict elimination diets supervised by the nutritionist. Main new therapeutic strategies for FA include allergen-specific (oral, sublingual, epicutaneous, subcutaneous immunotherapy and heat treatment of food) and non-allergen-specific therapies (humanized monoclonal antibodies, anti-IgE and anti-IL5, probiotics). An incorrect diagnosis is likely to result in unnecessary dietary restrictions, which, if prolonged, may adversely affect the child's nutritional status and growth.

Keywords Cow's milk allergy · Probiotics · Skin prick test · Atopy patch test · Oral food challenge · Elimination diet

Introduction

Food allergy (FA) is a major health issue in western societies affecting between 5 and 10 % of young children [1]. During the last decade in the United States pediatric FA diagnosis rates increased by 18 %, however it has not been determined whether these findings are related to increased awareness and reporting. In any case, the use of specific medical diagnostic codes for FA does represent a real increase of the disease [2]. It has been estimated that FA, in the United States alone, accounts for 30,000 emergency room visits and 150 deaths per year [3]. In Italy, the

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number of hospital admissions due to food-induced anaphylaxis doubled in the last 5 years [4].

Although any food can provoke a reaction, relatively few foods are responsible for the vast majority of significant food-induced allergic reactions in children: cow's milk, hen's egg, soy, wheat, peanuts, tree nuts, fish and shellfish [2, 5]. Allergy to other foods is increasing too, as exemplified by reports on sesame and kiwi [6]. There is also an increasing appreciation of oral allergy syndrome in children: patients with birch pollen allergy have cross reactions to heat-labile proteins in various fruits and vegetables [7].

Correct diagnosis of FA is important to accurately establish the prevalence and incidence of this condition and to ensure appropriate patient care. In fact, FA may have deleterious effects on family economics, social interactions, school and work attendance, and health-related quality of life. The diagnostic work-up in a child with FA includes many steps, but the essential criteria are a thorough medical history-taking and physical examination together with a clear response to the oral food challenge (OFC) [8•].

The Multistep Diagnostic Process

Food allergies can be broadly divided into IgE (type I hypersensitivity)- and non-IgE (usually type IV hypersensitivity)-mediated diseases; or mixed, involving other immunoglobulins, immune complexes and/or cell-mediated mechanisms. These differ in clinical presentation, diagnostic testing, and prognosis [8•]. IgE-mediated reactions are characterized by an acute onset of symptoms generally within 2 h after ingestion of or exposure to food. IgE-mediated reactions to food typically involve the skin, gastrointestinal tract, and respiratory tract and may include systemic reactions (anaphylactic shock). Non-IgE-mediated immunological reactions (e.g., cell-mediated) include food–protein-induced enterocolitis, proctocolitis, and enteropathy syndromes. These conditions primarily affect infants or young children who present with abdominal complaints, such as vomiting, abdominal cramps, diarrhea, and occasionally blood in the stool and failure to thrive or poor weight gain. Examples of FA co-morbidities with mixed IgE- and non-IgE-mediated causes include eosinophilic gastrointestinal diseases and atopic dermatitis [9]. Table 1 summarizes the main food-induced allergic disorders.

Adverse reactions to food that are not classified as FA include host-specific metabolic disorders (e.g., lactose intolerance), a response to a pharmacologically active component (e.g. an adverse reaction triggered by tyramine in aged cheeses), or toxins (e.g., food poisoning). Additionally, psychological (food aversion and anorexia nervosa) or neurologic (e.g., gustatory rhinorrhea from hot or spicy foods) responses can mimic FA [10•].

Food allergens may be split into two major classes: class 1, the major food allergens, and class 2, the indirect food allergens. Class 1 allergens comprise the classical food allergens to which patients become sensitized by the oral route or, possibly, through the skin. For these allergens, there is a clear association between ingestion (or contact) with the food and the onset of symptoms. Usually the allergens are heat stable; therefore both uncooked and cooked foods are problematic. Frequently, more than one organ system is involved. The primary allergens are highly conserved proteins or carbohydrates present within the pollen and fruits of a wide variety of plants. They include pathogenesis-related proteins (PRP), profilins, cross-reactive carbohydrate determinants (CCD) and lipid transfer proteins (LTP). Class 2 allergens comprise mainly pollen-derived allergens with cross-reactivity to foods and are often associated with oral allergy syndrome, sometimes called pollen-FA syndrome. Most commonly, sensitization is through the respiratory tract following pollen inhalation. Because class 2 allergens are usually heat labile and destroyed in the gastrointestinal tract, symptoms immediately follow eating raw fruit or vegetables. Examples of class 2 food allergens are latex, kiwi, apple, peach, hazelnut [10•, 11•].

The evaluation of a child with suspected FA includes: in-depth patient history, physical examination, screening tests, response to elimination diet and to OFC (Fig. 1). In children with multiple FAs, the response to elimination of single antigens is incomplete, and lengthy assessment with a very restricted diet is often required. The physician should obtain a detailed patient history focused on the kind and intake of symptom-inducing food, the time gap between food ingestion and onset of symptoms, reproducibility, presence or absence of any other symptom-inducing conditions, and the time of the last symptom. Timing of the first and last occurrences can reveal whether sensitivity is increasing or waning. An evaluation of possible cross reactions is helpful in selected cases. These considerations together with the quantity necessary to trigger a reaction are helpful for planning the best procedures to explore the presence of sensitization to particular foods and to perform OFC as well. Occasionally, the history can be complicated by the fact that trace amounts of foods may occur in certain products. The differential diagnosis could be particularly difficult in subjects with non-IgE-mediated FA where symptoms occur hours or days later. In addition, in a child with gastrointestinal symptoms, the differential diagnosis must also include: infections, gastrointestinal functional disorders, celiac disease, brush border enzyme deficiencies, cystic fibrosis and other primitive forms of pancreatic insufficiency, inflammatory bowel diseases, anatomical defects (e.g., pyloric stenosis, malrotation), metabolic disorders

Table 1 Main food-induced allergic disorders

	IgE-mediated/acute onset (onset time 30 min up to 2 h)	Non-IgE mediated/delayed onset (onset time few hours to days)	IgE- or non-IgE mediated/ delayed onset (onset time few hours to days)
Gastrointestinal tract	Oral allergy syndrome; gastrointestinal anaphylaxis	Dietary protein proctitis, colitis, enterocolitis, enteropathy; gastroesophageal reflux disease; food–protein-induced enterocolitis syndrome; chronic constipation	Eosinophilic esophagitis, gastroenteropathies
Respiratory tract	Rhinitis; conjunctivitis; asthma	Chronic pulmonary disease (Heiner syndrome)	Asthma
Skin	Urticaria; angioedema	Contact dermatitis	Atopic dermatitis
Systemic	Anaphylaxis; food-associated, exercise-induced anaphylaxis	–	–

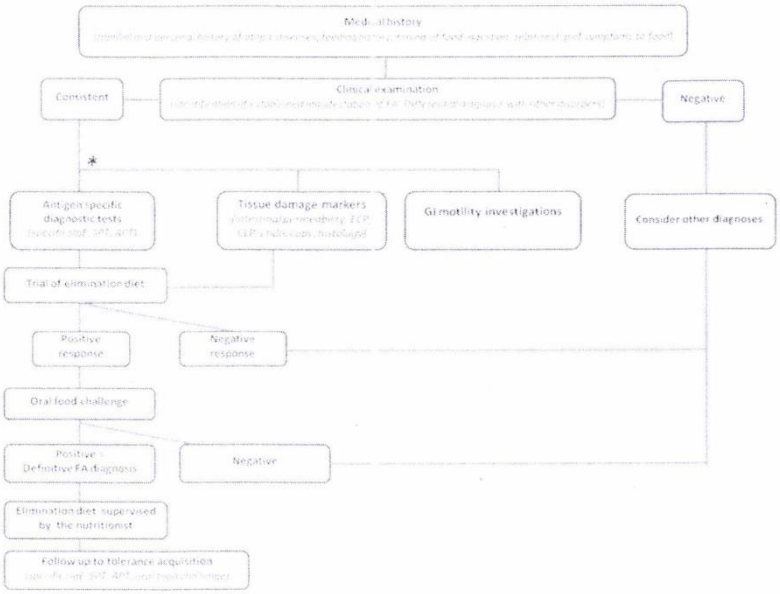

Fig. 1 The diagnostic algorithm for food allergy. *SPT* skin prick test, *APT* atopy patch test, *ECP* eosinophilic cationic protein, *CLP* fecal calprotectin, *FA* food allergy. *Tissue damage markers and GI motility investigations could be used in patients with gastrointestinal sings and symptoms possibly related to FA

(e.g., galactosemia), adverse reaction to drugs, and Munchausen syndrome/Munchausen syndrome by proxy.

Antigen-Specific Diagnostic Tests

In Vitro Tests

In vitro tests (determining serum-specific IgE antibodies against food allergens) are commonly used in the diagnostic workup of FA, especially in patients with severe atopic dermatitis or patients taking antihistamines, where skin tests are not suitable. Serum IgE levels can be assessed for crude allergen extracts, individual allergens, or even allergenic peptides. The results can be a simple qualitative measurement resulting in a yes or no answer for the presence of food-specific IgE or alternatively they may determine a quantitative antibody level [12•]. Measurement of allergen-specific IgE is performed using the radioallergosorbent test; the detection limit of the system usually is 0.35 kU/L IgE. A subject is considered sensitized if their specific IgE levels exceed the detection limit. Many clinical studies have been performed to evaluate the reliability of in vitro testing for food-specific IgE to clinical FA. Using the ImmunoCAP system, threshold levels of allergen-specific IgE to egg (6 kUa/L), peanut (15 kUa/L), fish (20 kUa/L), and milk (32 kUa/L) have been shown to portend positive OFC results with greater than 95 % accuracy [13•]. Therefore, evaluation of allergen-specific IgE could possibly obviate the need for potentially life-threatening reactions to foods during challenge tests. There

is emerging evidence that component-resolved diagnosis through measurement of specific IgE to individual, most often recombinant, food allergens may be superior to measurements of specific IgE to crude allergen extracts. For example, clinically relevant peanut allergy seems to correlate with the detection of specific IgE antibodies to Ara h 2, a seed storage protein in peanuts [14]. Homolog seed storage proteins also exist in tree nuts and seeds. In hazelnut allergies, detection of specific IgE to Cor a 9 suggests a FA that might result in a life-threatening reaction, whereas detection of specific IgE solely to Cor a 1, the homolog of the birch pollen allergen Bet v 1, suggests a pollen-associated FA [15]. Similarly, positive specific IgE to the LTP of hazelnut, Cor a 8, suggests an increased risk of severe systemic reactions. Similar improvement of the test performance and diagnostic sensitivity has been seen when using single kiwifruit allergens compared with the extract [16].

In IgE-mediated FA, circulating antibodies recognize specific molecular regions on the antigen surface (epitopes), which are classified according to their specific amino acid sequence (sequential or linear epitopes) or the folding and configuration of their protein chains (conformational epitopes). Subjects with transient FA produce IgE antibodies primarily directed at conformational epitopes (dependent on the protein's tertiary structure), whereas those with persistent FA also produce IgE antibodies against sequential epitopes, which are heat stable [17]. Greater IgE epitope diversity and higher IgE affinity are associated with more severe FA [18]. In the future, IgE epitope mapping has the potential to become an additional tool for the diagnosis/prognosis of FA and lead to a better understanding of the pathogenesis and tolerance induction [19].

Some cellular tests, i.e. tests determining the reactivity of blood cells in vitro (e.g., determination of histamine release, basophil degranulation, CAST-determination of sulfidoleukotrienes produced by IL-3 primed basophils stimulated by allergens in vitro- and flow cytometric basophil activation test), are increasingly used in selected tertiary centers with the aim to accurately define their diagnostic accuracy and standardization. Complementary/alternative tests (e.g. ALCAT-test, bioresonance, kinesiology, leukocytotoxic test, electrodermal tests, iridology and hair analysis) are quite popular in clinical practice, but there is absolutely no evidence of their diagnostic value [20, 21].

In Vivo Tests

Immediate hypersensitivity skin prick tests (SPTs) examine for the presence of food protein specific IgE. SPTs usually have sufficient sensitivity and specificity to be the sole method of skin testing necessary for most clinical scenarios. In general, SPTs have a sensitivity of ~90 % but a specificity of only about 50 % [22]. An important component of management is the understanding by clinicians of the predictive value of individual FA tests. The larger the size of the wheal on a skin test, the more likely a patient will react to the food (Table 2) [12, 22, 23]. The quality and allergen content of the extract employed for the test are pivotal. Thus, patients with oral allergy syndrome induced by fresh, but not cooked, tree fruit associated with tree pollen allergy usually do not show positive tests to commercial extracts. Some studies reported that the SPTs are positive 40 % of the time with commercial extracts and 81 % of the time using fresh foods. The overall concordance between a positive prick test and a positive challenge test is 59 % with commercial extracts and 92 % when fresh foods were used [24]. When a history is positive, and a commercial food antigen SPT is negative, a prick using fresh food should be considered.

For non-IgE-mediated disorders, fewer diagnostic tools exist. Atopy patch tests (APTs) are able to explore cell-mediate reactions, and they are the most used FA screening tests in the clinical practice. There is no minimum age for these tests, which can be performed also in preterm babies and infants with useful results [9, 25]. APTs have been proposed for the initial diagnostic approach in children with suspected non-IgE-mediated CMA and atopic dermatitis [26], gastrointestinal disorders [26–28] and eosinophilic esophagitis [27]. The use of APTs in the clinical practice of pediatric gastroenterology could be limited by subjective interpretation and intra-observer variation. Recently it has been demonstrated that in children with gastrointestinal symptoms the diagnostic accuracy of APTs is influenced by the severity of skin signs [29] and that APTs could be a valuable tool in the follow-up of pediatric patients with gastrointestinal symptoms related to non-IgE-mediated cow's milk allergy by contributing to the determination of whether an OFC can safely be undertaken [30].

Table 2 Diagnosis of food allergy with the use of 95 % positive predictive value (PPV) for specific IgE and skin prick tests

	Serum-specific IgE (U/mL)	Skin prick test wheal diameter (mm)
Cow's milk	15 (5 if the child age is <2 years)	8 (6 if the child age is <2 years)
Hen's egg	7 (2 if the child age is <2 years)	7 (5 if the child age is <2 years)
Fish	20	7
Peanuts	15	8 (4 if the child age is <2 years)

Tissue Damage Markers and Gastrointestinal Motility Investigations

Several procedures could be adopted in children with gastrointestinal symptoms possibly related to FA. These include endoscopy with histologic evaluation, esophageal pH monitoring; together with noninvasive tissue damage markers, such as intestinal permeability, faecal eosinophilic cationic protein and calprotectin measurement [8, 31]. Although these noninvasive tests would be convenient to detect an intestinal mucosal reaction to foods, no conclusive studies are available on the diagnostic accuracy of these tests, alone or in combination, in the approach to a child with suspected FA. Patients with allergic eosinophilic esophagitis or gastroenteritis have peripheral eosinophilia, and children with severe allergic eosinophilic gastroenteritis might have anaemia, blood in the stool and decreased serum albumin and IgG levels. Endoscopy with biopsies are the most definitive approach and might help in the differential diagnosis. Density >15 eosinophils/HPF in the oesophagus is diagnostic for allergic eosinophilic esophagitis, especially if the oesophageal pH monitoring is normal and there is lack of response to high-dose proton pump inhibitors medication [26]. If food-induced enteropathy or colitis are suspected, intestinal biopsies revealing primarily eosinophilic infiltration of the mucosa may be helpful. The mucosal lesions in FA enteropathies are characteristically focal. Thus, sampling error may result in negative biopsies in a discrete number of cases. Colonic biopsies are more often helpful in cases with allergic colitis, usually seen in infants with FA-induced hematochezia. In children with FA, electrogastrographic evidence of severe gastric dysrythmia and delayed gastric emptying during OFC have been demonstrated. The investigations on FA-related motility disorders could be performed by multichannel intraluminal electrical impedance testing, micromanometric techniques, gastroelectrophysiological studies, or measurement of gastric emptying by ^{13}C-octanoic acid breath test [32].

Oral Food Challenge

Oral food challenge still represents the gold standard for the diagnosis of FA in order to avoid unjustified diets. When the food considered for the challenge is still part of the patient's diet, a strict elimination diet should be prescribed for at least 2 weeks before the OFC. The optimal duration of elimination diet before OFC depends mainly on symptom severity. Different FA-related gastrointestinal diseases need different durations of elimination diet before OFC: an elimination of 4–6 weeks is considered adequate for enterocolitis, proctitis/proctocolitis and enteropathy. However, for gastroesophageal reflux disease and constipation, just 2–4 weeks are adequate. The OFC is done by feeding gradually increasing amounts of the suspected food under observation by a physician over a period of hours, protracted for days when no immediate reaction occurs. Because the procedure carries a small risk of anaphylaxis, it should be conducted in a supervised medical setting where resuscitation equipment is available. Several papers have been published recently on this topic aiming at standardizing the procedure [33–35]. The main problems of OFC are related to the wide variety of symptoms possibly related to immunological mechanism of FA that lead to difficulties in the interpretation of results and to the optimal timing and dosage of this procedure. A rather complex, double-blind-placebo-controlled food challenge (DBPCFC), routinely used in research, is recommended in clinical settings only when patients report entirely subjective symptoms; whereas an open OFC without placebo is commonly used in children under the age of 3 years and when objective symptoms are present [36–38]. Unfortunately, the diagnosis of FA is frequently incomplete, incorrect, or self-reported, and a correct diagnostic work-up, confirmed by OFC, seems to be adopted in only a minority of cases [39].

Therapeutic Options

Elimination Diet

Although numerous therapeutic treatment options are currently being investigated, dietary avoidance remains the primary treatment for FA [40•]. Inadequate nutritional status, growth, and dietary intakes have been demonstrated in children with FA [41]. Altered growth status may be due to potential loss of nutrients caused by continued allergic inflammation and/or abnormal intestinal permeability caused by noncompliance with the diet, unbalanced diet and additional undiagnosed FA or inappropriate substitute foods [41]. This highlights the need to make every effort to optimize nutrition because inadequate nutrient intake may worsen the risk of lower growth rates in this population. The elimination diet should be considered carefully depending on: mechanism of FA; symptoms; nutritional status; and concomitant factors like food aversive behavior. A properly managed, well-balanced elimination diet prescribed with the help of certified dietitians, can lead to resolution of symptoms while maintaining or even optimizing nutritional status.

Critical points in the management of the patient are the protein:energy ratio and the energy requirements. In fact, the rate of catch-up growth required (based on the weight for height or S.D. scores) depends on the individual patients' current nutritional status, and allergen restrictions. The ideal protein:energy ratio lies between 8.9 and 11.5 % of total energy; this ratio could be further increased depending on

the level of stunting. In addition, for optimal catch-up growth, 5–10 g/kg/day of protein and 105–126 kcal/kg/day are commonly required [42].

An often neglected component of treatment is to ensure patients and/or their caregivers understand the importance of complete adherence to the diet, as inadvertent consumption of an offending food can prevent resolution of symptoms and render challenge results useless. As many FAs of early childhood resolve over time, regular reassessment by the allergist is also important to avoid extended, unnecessary elimination diets.

Even under the best circumstances, avoidance of allergens is not simple. The variety of commercial food items available is ever expanding, and ingredients in commercial products change frequently, requiring consumers to read product labels each and every time an item is purchased. Laws that guide the labeling of food allergens vary from country to country [43]. These laws typically require identification and disclosure on product labels of those food components that are considered 'common food allergens' or 'major allergens'. Healthcare practitioners and consumers should be aware of their country's food allergen labeling laws. When traveling abroad, consumers with FA should always check the food allergen labeling laws of their destination country prior to purchasing and consuming packaged foods [44].

Immunotherapy

The main new therapeutic perspectives for the treatment of FA include allergen-specific (oral, sublingual, epicutaneous, subcutaneous immunotherapy (SCIT) and heat treatment of food) and non-allergen-specific therapies (humanized monoclonal antibodies, anti-IgE and anti-IL5, probiotics). Oral food immunotherapy (OIT) is currently the most investigated approach for persistent FA. This method is based on the concept that repeated oral/intestinal exposures to antigens normally lead to tolerance. OIT protocols usually provide an initial stage with progressive increase of the dose, followed by a phase of slow accumulation to achieve the desired maintenance dose. Several studies have demonstrated that OIT with milk is effective in desensitizing patients with cow's milk allergy [45, 46]. The aim of the experimental studies of OIT is to develop a safe protocol that can be used in routine clinical practice. There is still controversy on OIT, due to concerns for heterogeneity in protocols, compliance of patients and their families, failure of desensitization and presence of atopic disease. Moreover, patients with complicated IgE- and non-IgE-mediated disease may not respond well to OIT. In sublingual immunotherapy (SLIT) the food is administered sublingually, held in the mouth for few minutes, and then spat or swallowed. Several studies with hazelnut, milk, peanut, and peach have demonstrated the benefit of SLIT in increasing the amount of the food tolerated on DBPCFC [47–49]. Side effects are generally mild, mainly limited to oropharyngeal symptoms, and rarely require oral antihistamine administration. However, the maximum dose that can be administered sublingually is limited, which may limit the maximum dose of food that can be ultimately tolerated [50•]. Further studies are needed to standardize the method and demonstrate its safety in larger numbers of patients. The use of SCIT in persistent FA was quickly discontinued after reports of fatal reactions with peanut injections. Consequently, this approach is no longer used [50•]. In epicutaneous immunotherapy (EIT), patients receive three 48-h skin patch applications (1 mg of skimmed milk powder) per week for 3 months [51•]. Adverse effects are mostly local cutaneous reactions and discomfort (pruritis and eczema), but don't include any severe systemic reactions. While EIT appears safe, additional studies are required to examine efficacy in terms of additional foods, and what are the maximum doses that can be applied epicutaneously and tolerated orally [50•]. Omalizumab is a recombinant humanized monoclonal IgE-blocking antibody. It's an allergen non-specific modality of FA treatment. It decreases or prevents the allergic response triggered by IgE molecules, binding to the constant domains of free circulating IgE molecules, reducing IgE-mediated mast cell and basophil degranulation on allergen exposure [52•, 53]. Subcutaneous injections of omalizumab have been shown to have relatively few and tolerable side effects mainly at the injection site. Less common reactions included bronchospasm, hypotension, syncope, urticaria, angioedema and rarely anaphylaxis [54, 55]. Other therapeutic strategies with modified allergens (peptides, sequences of oligodeoxynucleotides, plasmid DNA) have been evaluated in preclinical studies for the possible treatment of peanut allergy, but they are currently underutilized. It is possible that in the future such studies can be resumed as a result of a better characterization of antigenic epitopes responsible for the various forms of FAs.

New Therapeutic Perspectives Deriving from Immunonutrition

"Immunonutrition", a diet-induced immunomodulation, is becoming an increasingly realistic therapeutic option for FA. The more promising strategies involve modified antigenic peptides and probiotics. Heating cow's milk and hen's egg decreases protein allergenicity by destroying conformational epitopes. The introduction of extensively heated milk and egg protein in the diet of subjects with milk and egg allergy, who tolerate the baked form, is becoming an alternative approach to induce a faster acquisition of oral tolerance. Children who incorporated

baked milk or baked egg into the diet are 16 and 14.6 times more likely to become tolerant to unheated milk and egg compared with a comparison group of children who continued strict avoidance of these foods [56•, 57•].

Recent data strongly suggest that gut microbiota is important for oral tolerance development [58•]. Administration of the probiotic *Lactobacillus rhamnosus* GG (LGG) to food-allergic children (age <2 years, challenge-proven and affected by mild-to-moderate eczema) improved the eczema score significantly [58•]. Studies in infants with eczema who received formulas supplemented with LGG showed benefits in decreasing gastrointestinal symptoms [59]. For instance, after a challenge study in infants allergic to cow's milk proteins, fecal IgA levels were detected to be higher, and TNF-α levels were lower in the LGG applied group compared to the placebo [58•]. Nermes et al. [60] showed a significant decrease in IgA- and IgM-secreting cells in infants with atopic dermatitis treated with extensively hydrolyzed casein formula (eHCF) supplemented with LGG, suggesting that this particular probiotic is able to enhance gut barrier function and accelerate immunological maturation in infants with FA. Moreover, LGG is able to induce IFN-γ secretion in infants with CMA and in infants with IgE-associated dermatitis, but not in infants without CMA [58]. The addition of LGG to an eHCF significantly improved the recovery of the inflamed colonic mucosa if compared to that obtained with eHCF alone in infants with blood in the stool and CMA-induced colitis, as indicated indirectly by greater decrease in fecal calprotectin and in the number of infants with persistence of occult blood in stools after 1 month [61]. We recently demonstrated that an eHCF containing LGG is able to accelerate the development of tolerance acquisition in infants with CMA. Infants (aged 1–12 months), consecutively referred for suspected CMA but still receiving cow's milk proteins, were invited to participate in the study. Subjects were randomly allocated to one of the two groups of dietary interventions: a control group, who received an eHCF; and an active group, who received an eHCF containing LGG (at least 1.4×10^7 CFU/100 mL). After 12 months, the DBPCFC was negative in 15 of 28 control infants (53.6 %) and in 22 of 27 infants receiving eHCF with LGG (81.5 %, $p = 0.027$). These findings suggest an innovative approach for infants affected by CMA, namely an "active dietotherapy" able to induce a faster symptom's remission and to reduce the time of tolerance acquisition [62•].

Conclusions

Correct diagnosis of FA is crucial to ensure appropriate patient care. The essential criterion is a clear response to elimination diet, and other diagnostic tests are secondary to this. OFC plays a crucial role in the diagnostic approach to a child with suspected FA, but it is largely underutilized. Potential responsible factors contributing to the lack of a correct diagnostic work-up in the vast majority of cases could be numerous, and include lack of training on the procedure, increased reliability on screening methods, extensive time needed, fear of risk, and suboptimal fee reimbursement. A comprehensive nutrition assessment with appropriate intervention is warranted in all children with FAs to meet nutrient needs and optimize growth. Frequently an elimination diet is absolutely necessary to prevent potentially life-threatening food allergic reactions. However, dietary elimination in FA may also have undesirable consequences. An increasing number of evidences suggest the role of selected probiotics in prevention or treatment of FA. These data support the importance of a "nutritional immunology approach" able not only to efficiently cure the symptoms, but also to accelerate tolerance acquisition in children with FA.

Disclosure Roberto Berni Canani, Rita Nocerino, Vincenza Pezzella, Ludovica Leone, Tommaso Cozzolino, Rosita Aitoro, Lorella Paparo, Margherita Di Costanzo, Linda Cosenza, and Riccardo Troncone declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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2. Potential beneficial effects of butyrate against food allergy

Food allergy rates in children have rapidly increased with significant direct medical costs for the health care system and even larger costs for families with a food-allergic child. during the last decade, we observed a changing pattern in CMA with an increased prevalence, severity of clinical manifestations and risk of persistence until later ages in Western countries (111). Over the last twenty years rates of potentially life threatening reactions to food (anaphylaxis) have steadily risen in the developed world (99). For all these reasons, there is a strong need to develop effective strategies to stimulate oral tolerance acquisition and maintenance. The possible causes of food allergy become the target of intense scrutiny in recent years(112). Increasing evidence underline the importance of gut microbiome in the development of allergic diseases. It has been demonstrated that in early life the gut microbiome influence immune development, balance of Treg cells and bacterial metabolites which may increase the risk of food allergy. The colonizing bacteria originate mainly from the mother‘s gut and vaginal tract (113). After delivery, breast feeding continues to enhance the original inoculum by the introduction of specific lactic acid bacteria, Bifidobacteria and other bacteria from the mother‘s skin. These bacteria set the basis for intestinal microflora development and modulation. An imbalance in the compositional configuration of the gut microbiota, dysbiosis, alters the host-microbiota homeostasis, which is a requisite for the development and function of immune cells in the gut associated lymphoid tissue. The importance of this reciprocal regulation of the microbiota and immune system culminates in early infancy, when the balance between homeostasis and inflammation programs later disease risk. In particular, early exposure to commensal bacteria plays a crucial role in Th1/Th2 polarization and proper immune regulatory mechanisms. Regulatory T cells

(Tregs), that express the transcription factor Foxp3, are also critical for the induction and the maintenance of food oral tolerance and for the regulation of intestinal inflammation.

Short chain fatty acids (SCFAs) such as propionate, acetate and butyrate are gut microbiota derived bacterial fermentation products that selectively expand Tregs in the large intestine (114). These SCFAs stimulate the expansion and immune suppressive properties of Tregs, such as the production of IL-10 (115). Among the SCFAs, butyrate has received particular attention for its multiple beneficial effects from the intestinal tracts to the peripheral tissues. The mechanisms of action of butyrate are multiple and involve also an epigenetic regulation of gene expression through the inhibition of histone deacetylase. In particular, the inhibition of histone deacetylase 9 and 6 increases Foxp3 gene expression, as well as the production and suppressive function of Tregs(116). The identification of bacterial metabolites, that affect host immunity, may be an interesting strategy to prevent and/or to treat food allergy and promote human health.

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Chapter 5

POTENTIAL BENEFICIAL EFFECTS OF BUTYRATE AGAINST FOOD ALLERGY

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ABSTRACT

The intestinal immune system has coevolved with gut microbiota for the maintenance of body health. An imbalance in gut microbiota composition, named dysbiosis, has been associated with various gastrointestinal and extraintestinal diseases, including food allergies. Gut microbiota is a crucial factor for food oral tolerance and it regulates an appropriate balance between immune effectors and regulatory pathways. Regulatory T cells (Tregs), that express the transcription factor Foxp3, are also critical for the induction and the maintenance of food oral tolerance and for the regulation of intestinal inflammation. Short chain fatty acids (SCFAs) such as propionate, acetate and butyrate are gut microbiota derived bacterial fermentation products that selectively expand Tregs in the large intestine.

These SCFAs stimulate the expansion and immune suppressive properties of Tregs, such as the production of IL-10.

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Among the SCFAs, butyrate has received particular attention for its multiple beneficial effects from the intestinal tracts to the peripheral tissues.

The mechanisms of action of butyrate are multiple and involve also an epigenetic regulation of gene expression through the inhibition of histone deacetylase. In particular, the inhibition of histone deacetylase 9 and 6 increases Foxp3 gene expression, as well as the production and suppressive function of Tregs.

The identification of bacterial metabolites, that affect host immunity, may be an interesting strategy to prevent and/or to treat food allergy and promote human health.

THE CHANGING PATTERN OF FOOD ALLERGY

Food allergy (FA) is a major health issue in Western countries with a substantial effect on quality of life of both patients and their relatives. Some evidence has suggested that the prevalence of the disorder in childhood has increased in recent years, affecting between 5 and 10% of young children in Western countries [1]. During the last decade in the United States pediatric FA diagnosis rates increased by 18%. However, it cannot be determined how much of the increases in estimates are truly attributable to increases in clinical disease and how much are attributable to increased awareness by physicians, other health care providers, and parents. However, the consistent increases across surveys and among children in all age, gender, and race/ethnicity groups provide evidence that the increases are not limited to a certain setting, reporting mechanism, or demographic group [2]. Although any food can provoke a reaction, relatively few foods are responsible for the vast majority of significant food induced allergic reactions in children: cow's milk, hen's eggs, soy, wheat, fish, peanuts, and shellfish [3]. Features common to major food allergens are that they are water soluble glycoproteins ranging from 10 to 70 kDa in size and are relatively stable to heat, acid and proteases [4]. Cow's milk allergy (CMA) is the most common FA in early childhood, with an estimated incidence ranging between 2% and 3% in infants and marginally lower in older children [5]. The majority of children will regain tolerance to cow's milk proteins (CMP) within the first 5 years of life [6]. However during the last decade, we observed a changing pattern in CMA with an increased prevalence, severity of clinical manifestations and risk of persistence until later ages in Western countries [7]. Over the last twenty years rates of potentially life threatening reactions to food (anaphylaxis) have steadily risen in the developed world [8]. For all these reasons, there is a strong need to develop effective strategies to stimulate oral tolerance acquisition and maintenance.

FOOD ORAL TOLERANCE AND FOOD ALLERGY

Food antigens and intestinal microflora constitute the majority of the antigen load in the intestine, and the "default" reaction of the immune system confronted with them leads to systemic unresponsiveness. This phenomenon is known as oral tolerance and is a key feature of intestinal immunity [9]. The systemic unresponsiveness to dietary antigens is induced in mesenteric lymph nodes as a result of inactivation of antigen specific T cells when antigen loaded dendritic cells (DCs) seed antigens from the intestinal mucosa [10]. A subset of DCs

mediates the selective induction of Treg cells in response to an antigen encountered in the gastrointestinal mucosa.

CD103+ DCs are migratory and traffic to the mesenteric lymph nodes where gut intraluminal antigens are presented by CD103+ DCs [11]. These DCs also induce the development of Treg cells through mechanisms dependent on factors such as anti-inflammatory cytokine transforming growth factor–β (TGF-β), which is necessary for the development of food oral tolerance [12]. In a broader view, the complex interaction between intestinal contents and immune and non-immune cells result in an environment that favors the tolerance by the induction of IgA antibodies and CD4+ T regulatory cells (producing IL-10 and IFN-γ) [13]. This ensures that a homeostatic balance is maintained between the intestinal immune system and its antigen load, so that it retains the ability to recognize dangerous and harmless antigens as foreign and preserves the integrity of the intestinal mucosa. The inappropriate immune responses to food and intestinal microflora that are responsible for FA are a result of a deregulation of these crucial processes [14]. An allergic reaction mainly corresponds to the activation of Th2 cells against food allergens and occurs in two phases: the first phase corresponds to transport of the allergen through the intestinal barrier, its capture by antigen presenting cells (DCs or enterocyte), and its presentation to naive Th0 cells, which differentiate in the presence of IL-4 into Th2 cells. Activated Th2 cells then produce an IL-4 cytokine that enables the production of allergen-specific IgE by B cells [15]. These secreted IgEs then bind to mast cells via the IgE receptor FcεRI. The activation phase corresponds to the degranulation of mast cells after further exposure to the same allergen that links directly with specific IgE on the surface of these cells. This phenomenon triggers a release of the allergic mediators involved in clinical manifestations of allergy. In addition to acute allergic reactions triggered by IgE-mediated immune responses to food proteins, there are cell-mediated manifestations. Non-IgE mediated reactions or cell mediated responses include those reactions in which specific cells, different from mastocytes (and basophils), are responsible for the allergic reaction, and they mostly involve the gastrointestinal tract; mixed IgE mediated and cell mediated responses are those reactions in which both IgEs and immune cells are involved. The symptoms of food allergies may vary according to age: in early childhood, gut and skin involvement is common, whereas respiratory and the oral allergy syndrome are more frequent in older children and adults [16].

Recent Evidences in the Pathogenesis of Food Allergy

Microbial gut colonization begins after birth and this process is affected by the newborn infant's gestational age, mode of delivery and first feeding strategies. The colonizing bacteria originate mainly from the mother's gut and vaginal tract [17]. After delivery, breast feeding continues to enhance the original inoculum by the introduction of specific lactic acid bacteria, Bifidobacteria and other bacteria from the mother's skin. These bacteria set the basis for intestinal microflora development and modulation. An imbalance in the compositional configuration of the gut microbiota, dysbiosis, alters the host-microbiota homeostasis, which is a requisite for the development and function of immune cells in the gut associated lymphoid tissue. The importance of this reciprocal regulation of the microbiota and immune system culminates in early infancy, when the balance between homeostasis and inflammation

programs later disease risk. In particular, early exposure to commensal bacteria plays a crucial role in Th1/Th2 polarization and proper immune regulatory mechanisms.

Germ free animals do not develop oral tolerance and maintained a T helper 2 type immune response to orally administered ovalbumin. This could be corrected by the reconstitution of the microbiota at the neonatal stages, but not any reconstitution implemented at a later ages [18]. These findings documented a decisive role of the gut microbiota for the acquisition of food oral tolerance in early life.

Exposure to a normal intestinal microflora in early life allows for a change in the lymphocyte T-helper 1/lymphocyte T-helper 2 balance, favoring a T-helper 1 cell response [19]; while an imbalance in the compositional configuration of the gut microbiota, dysbiosis, alters the host-microbiota homeostasis, producing a shift of the T-helper 1/T-helper 2 cytokine balance toward a T-helper 2 response and a consequent activation of T-helper 2 cytokines with an increased production of immunoglobulin E [20]. Imbalance in intestinal microbiota composition have been documented in patients with food allergy. Recently, Nakayama J et al. [21] profiled the fecal bacteria compositions in allergic and non-allergic infants by using the 16S rRNA gene short-tag pyrosequencing approach and correlated some anomalies in the microbiota with allergy development in later years. The comparative analysis of genus-level composition data identified population differences in some genera between the allergic and non-allergic groups. Interestingly, allergic infants who showed high colonization of Bacteroides and/or Klebsiella showed less colonization of Clostridium perfringens/butyricum, suggesting antagonism between these bacterial groups in the gastro-intestinal tract. This finding is different from that of previous studies in which Clostridium was more abundant in allergic infants [22-24]. This discrepancy may be attributable to species differences, because Clostridium contains a highly diverse group of bacteria.

Recently, a new link between dysbiosis and food allergy development has been provided. Maternal use of antibiotics before and during pregnancy was associated with an increased risk of cow's milk allergy in the offspring and the risk of cow's milk allergy increased with increasing number of child's antibiotics used from birth to diagnosis [25]. Antibiotics alters intestinal eubiosis and several intestinal microbiota-associated characteristics, such as short-chain fatty acid (SCFA) pattern. In fact, patients with antibiotic-associated diarrhoea showed an imbalance in gut microbiota and the amounts of faecal SCFAs were reduced [26].

In addition to antibiotic exposure, there are a range of factors in the modern environment that may be associated with changes to both the microbiome and risk of FA, such as mode of delivery, infant feeding practices, farming environment and country of origin. The role of the prenatal maternal environment in early immune programming is also important. In particular, the effects of maternal microbial exposure on offspring are multifaceted, including direct effects on maternal immune system and the more obvious effects on postnatal colonization of the infant.

The Role of Clostridia Species in the Protection against Food Allergy

CD4+ T regulatory cells (Tregs), which express the Foxp3 transcription factor, play a critical role in the maintenance of immune homeostasis and oral tolerance. Atarashi K. et al.

[27] showed that in mice the spore-forming component of indigenous intestinal microbiota, particularly clusters IV and XIVa of the genus Clostridium, promotes Treg cell accumulation in the colonic mucosa. Colonization of mice by a defined mix of Clostridium strains provided an environment rich in TGF-β and affected Foxp3+ Treg number and function in the colon. In a subsequent study, Atarashi K. et al. [28] isolated 17 strains within Clostridia clusters XIVa, IV and XVIII from a human faecal sample and demonstrated that these strains affect Treg cell differentiation, accumulation and function in the mouse colon (Table 1). Oral inoculation of Clostridium during the early life of conventionally reared mice resulted in resistance to colitis and downregulation of systemic immunoglobulin E responses in adult mice, suggesting a new therapeutic approach to autoimmunity and allergy.

BUTYRATE AS A PROTECTIVE FACTOR AGAINST FOOD ALLERGY

Clostridia species belonging to cluster IV and XIVa are the prominent source of acetate, butyrate, and to a lesser extent propionate in the colon [28]. Smith et al. [29] observed that feeding germ free mice three short-chain fatty acids (propionate, acetate, butyrate), individually or in combination, increased the number of Foxp3 Treg cells in the large intestine to an amount similar to that observed in conventionally reared animals (Figure 1). The majority of the expanded Treg cell population expressed the transcription factor Helios, which suggests that they acquired Foxp3 expression in the thymus. This is of particular interest because bacterial-driven Treg cell generation in the intestine is thought to occur locally and result in Treg cells that do not express Helios. This suggests that there are distinct pathways through which intestinal bacteria can influence intestinal Treg cells.

Table 1. Isolated Treg-cell inducing strains

Closest species/strain	Corresponding strain number
Clostridium asparagiforme	Strain 15
Anaerotruncus colihominis	Strain 13
Clostridiaceae JC13	Strain 8
Clostridium bolteae	Strain 7
Clostridiales 1_7_47FAA	Strain 28
Lachnospiraceae 7 1 58FAA	Strain 3
Clostridium scindens	Strain 26
Clostridium 7 3 54FAA	Strain 16
Ruminococcus sp. ID8	Strain 14
Clostridium indolis	Strain 9
Eubacterium fissicatena	Strain 21
Clostridium ramosum	Strain 18
Lachnospiraceae 3_1_57FAA	Strain 29
Clostridium sp. 14774	Strain 1
Lachnospiraceae 3_1_57FAA	Strain 27
Blautia product	Strain 6
Clostridium hathewayi	Strain 4

Note: The composition of the gut microbiota was analysed by 16S ribosomal RNA (rRNA) gene amplicon sequencing using a 454 sequencer (see reference [16]).

A combination of clostridia species (cluster IV and XIVa) stimulates local differentiation of Treg cells, whereas administration of short-chain fatty acids leads to accumulation of thymus-derived Treg cell populations [30].

The mechanisms of action of butyrate are multiple, but many of these involve an epigenetic regulation of gene expression through the inhibition of histone deacetylase. The inhibition of histone deacetylase 9 and 6 increases Foxp3 gene expression, as well as the production and suppressive function of Tregs [31].

The identification of bacterial metabolites, that affect host immunity, may be an interesting strategy to prevent and/or to treat food allergy and promote human health.

CLINICAL FINDINGS

Recently, we demonstrated that treatment of CMA infants with an extensively hydrolyzed casein formula (eHCF) supplemented with the probiotic *Lactobacillus rhamnosus* GG (LGG) accelerates oral tolerance acquisition to cow's milk [32, 33]. Subsequently, we tested the hypothesis that eHCF plus LGG induced effect on oral tolerance thanks to an influence of this dietary intervention on the composition of the gut microbiota [34, submitted].

High throughput sequencing technology (16S rRNA-based sequence analysis) was used to compare fecal samples from newly diagnosed CMA infants, collected before and after treatment with eHCF plus LGG, to those obtained from controls. Moreover, production of the SCFA butyrate was assessed in the same stool samples by gas chromatography. Treatment with eHCF plus LGG expanded gut microbiota populations associated with immunoregulatory effects and significantly increased butyrate production at intestinal level ($p<.05$). Statistically significant positive correlations were found between fecal butyrate concentration and the abundance of four clostridial genera: Faecalibacterium, Blautia, Roseburia, and

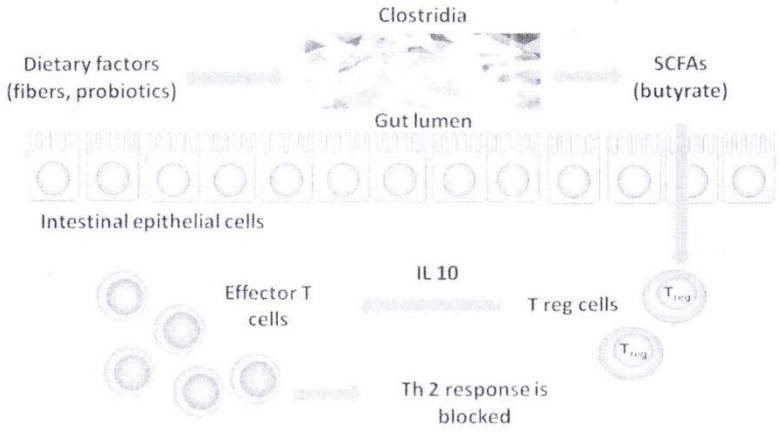

Figure 1. Intestinal microbiota that metabolizes dietary fibers can generate short chain fatty acids (SCFAs), such as butyrate, that enforce regulatory T cells in the gut.

Coprococcus ($p<.05$). All four genera increased in CMA infants after treatment with eHCF plus LGG. The protective effects of butyrate were also explored in a mouse model of CMA (5 week old female C3H/HeOuJ mice). Oral butyrate treatment (20 mg/kg/day) alleviates the allergic response in β-lactoglobulin sensitized mice, as demonstrated by a significant inhibition of acute allergic skin reaction, anaphylactic symptom score, body temperature decrease, intestinal permeability increase, and specific-IgE production ($p<.05$). Our data suggests that eHCF containing LGG promotes oral tolerance, in part, through its influence on the gut microbiome (Figure 2). These findings suggest a potential innovative therapeutic approach for infants affected by CMA, based on the effect of bacterial metabolites on host immunity and human health.

CONCLUSION

Food allergy rates in children have rapidly increased with significant direct medical costs for the health care system and even larger costs for families with a food-allergic child. The possible causes of food allergy become the target of intense scrutiny in recent years [35]. Increasing evidence underline the importance of gut microbiome in the development of allergic diseases. It has been demonstrated that in early life the gut microbiome influence immune development, balance of Treg cells and bacterial metabolites which may increase the risk of food allergy.

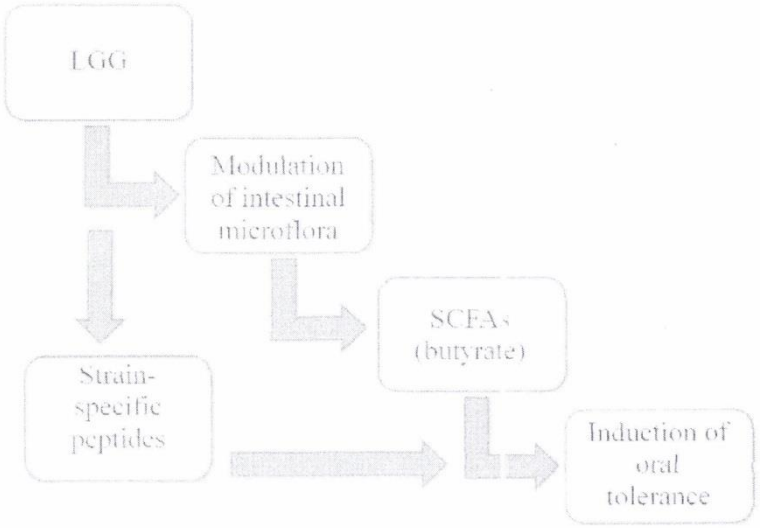

Figure 2. A schematic representation of probiotic mechanisms of actions, which explain the potential benefits of probiotics in allergy prevention and treatment.

There are a range of factors in the modern environment that may be associated with changes to both the gut microbiome and risk of FA, such as mode of delivery, antibiotic exposure, infant feeding practices, farming environment and country of origin.

Knowledge of the relationship between early life gut microbiome and allergic diseases may facilitate development of novel preventive and treatment strategies.

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2. An animal model to explore the mechanism of action elicited by Extensivly Hydrolized Casein Formula on cow's milk allergy

During the last decade, we observed a changing pattern in CMA with an increased prevalence, severity of clinical manifestations and risk of persistence until later ages in Western countries (111). In Italy, CMA is responsible for 42% of food-induced anaphylaxis in the pediatric population (99). For all these reasons, there is a strong need to develop effective strategies to stimulate oral tolerance acquisition and maintenance.

Extensively hydrolyzed casein formula (EHCF) has been proposed for prevention and treatment of CMA. For infants with CMA, we have previously demonstrated that eHCF induces a faster oral tolerance acquisition if compared with other dietetic choices (hydrolyzed rice formula, soy formula and amino acid based formula) and that this effect is up-regulated by the addition of the probiotic Lactobacillus rhamnosus GG (LGG, Nutramigen LGG) (117-118). The exact mechanism of these effects are still largely unknown. Preliminary evidences suggested a possible immunoregulatory role elicited by small peptides derived from bovine casein (119-120).

I aimed to investigate the mechanisms elicited by Extensivly Hydrolized Casein Formula (EHCF) and EHCF plus LGG in stimulating oral tolerance. To do this we used a well-established animal model of CMA using a major antigenic peptide from bovine milk, beta-lactoglobulin (BLG) (121). Two consecutive research phases were performed to explore the mechanism of action elicited by EHCF and EHCF plus LGG on CMA prevention and treatment.

3.1 Animal model in food allergy

Animals models are being used to understand of IgE-mediated disease and to determine the allergenicity of novel proteins. Several animal models have provided important information for understanding some of the mechanisms of allergenicity. Examples of animal models include the mouse, rat, guinea pig, and dog. As in humans, animal models have an innate tendency to develop tolerance to the myriad of proteins ingested, and it is difficult to generate valid food allergy models. A number of methods have been introduced to bypass the state of tolerance and initiate a food hypersensitivity that reflects human IgE-mediated food allergy.

The degree of sensitization should take into consideration the concentration of the allergen (high doses are known to induce tolerance); the allergen should be taken in context with the food source; the route (feeding and/or gavage are the recommended avenues) and duration of allergen exposure. And the age of the animal.

Other considerations include the genetic predisposition (high and low IgE responders), the use of adjuvants and the isotype specificity response. In addition the allergenicity profile should be comparable to what exists in the human response to different allergens, for example, anaphylactic episodes induced by peanut allergens to oral/pharyngeal symptoms elicited by fruit and vegetable allergens in the oral allergy syndrome. The simple production of IgE and binding of IgE to relevant proteins should be considered insufficient without evidence of histamine release using in vitro tests to support cross linking of IgE to bound mast cells and basophils.The mouse model is the most representative in literature. Inbred strains of mice have been characterized as being either high or low IgE-responder animals for both inhalant and food allergens.

As in humans, two separate events are required: the first event is a sensitization phase and, in the case of mice, the production of two anaphylactic antibodies, IgE and IgG1; the second phase is characterized as the allergic challenge following re-exposure to the allergen at the site of the response. In both inhalant and ingestant sensitizations, short-term daily exposures over a period of days or long-term exposure once a week over several weeks have been shown to induce allergen-specific IgE/IgG1. Differences in strain MHCs have been suggested as a likely reason for differences in mouse models' responses to different allergens, which may parallel a genetic predisposition in humans.

Cow's milk allergy (CMA) is a major cause of a transient food hypersensitivity in children. It involves the skin, respiratory tract, and gastrointestinal tract and can lead to systemic anaphylactic shock. In a study by Li et al. (122),6 several strategies were used to overcome oral tolerance in a mouse model and induce IgE-mediated cow's milk hypersensitivity. Three-week-old C3H/HeJ female mice were sensitized intragastrically with cow's milk plus cholera toxin as an adjuvant and were boosted five times at weekly intervals. Six weeks after the initial sensitization dose, mice were fasted and intragastrically challenged with two doses of CM 30 minutes apart. Hypersensitivity responses were assessed based on symptom scores, vascular leakage, plasma histamine release, PCA, serum antibody titers, skin testing, and histological examination. Symptom scores were identified by independent observation of the physical features.

The model exhibited characteristics of IgE-mediated cow's milk-induced food allergy. Elevated allergen-specific IgE levels were shown to be associated with systemic anaphylaxis, whereas levels of IgG1 were not; PCA reactions induced by serum from sensitized mice were eliminated by heating; and significant plasma histamine from mast cell degranulation was in evidence, all of which are important features of IgE-mediated food allergy. Serum casein levels after oral challenge were consistent with intestinal permeability

studies and histologic examination revealed changes in both the GI and respiratory systems. The model was regarded to be useful for evaluating mucosal and systemic immunopathogenic mechanisms involved in IgE-mediated cow's milk allergy.

Mice could prove valuable with respect to strain and allergen specificity in which related reactions may provoke information on the genetic basis of food allergen sensitization.

3.2 Extensivly Hydrolized Casein Formula (EHCF) and EHCF plus LGG in stimulating oral tolerance

The bioactive peptides generally contain 3–20 amino acids. The C- or N-terminal fragments are crucial for their activities. Activities are shown on the digestive system, the immune system, the cardiovascular system, the nervous system and body defense. Many of the bioactive peptides are derived from milk proteins. Examples are β-casomorphin (opiate activity), casein macro peptide (stimulation of release of CCK), β-casein fragments (angiotensin-I-converting enzyme inhibition), casein phosphopeptides (enhancement of mineral absorption), α-lactalbumin fragments (immune stimulation) and a wide range of antimicrobial peptides derived from caseins and whey proteins (123). Recently was report the identification of a peptide from yoghurts with promising potential for intestinal health: the sequence (94-123) of bovine β-casein. This peptide, composed of 30 amino acid residues, maintains intestinal homoeostasis through production of the secreted mucin MUC2 and of the transmembrane-associated mucin MUC4 (124).

Intestinal goblet cells are highly polarized secretory cells that reside throughout the length of the small and large intestine. They are responsible for production of the protective mucus coat by releasing the secreted mucin MUC2, a high-molecular-weight glycoprotein that is

stored within granules in their apical compartment. Mucus lubricates the intestinal surface, limits passage of luminal molecules into the mucosa, functions as a dynamic defensive barrier against enteric pathogens and acts as a substrate and a niche which the commensal flora can colonize (125).

Probiotic bacteria are proposed to benefit human health mainly by our general mechanisms of action. First of all, probiotics can clearly exclude or inhibit pathogens, either through direct action or through influence on the commensal microbiota (126). Certain probiotic strains have the capacity of to enhance the epithelial barrier function by modulating signaling pathways, such as nuclear factor-B (NF-kB), Akt and mitogen- activated protein kinase (MAPK)- dependent pathways, which lead to for example the induction of mucus(127), or increased tight junction functioning (128). Moreover, most probiotic strains can also modulate host immune responses, exerting strain-specific local and systemic effects(129). Many of the interactions between probiotic bacteria and intestinal epithelial and immune cells are thought to be mediated by molecular structures, known as microbe-associated molecular patterns (MAMPs), which can be recognized through specific pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (130)

Lactobacillus GG is known to modulate immune functions via various pathways, including those involving enterocytes, monocytes, mast-cells, dendritic cells, and regulatory T cells. Lactobacillus GG may alters the generation of cytokines involved in IgE-mediated CMA, and thereby can positively modulate the major pathways involved in CMA pathogenesis(131).

3.3 CMA Prevention

The design of the experiments is reported in Figure 8. Briefly, two weeks prior to sensitization period, mice were given three different experimental diets: control solid "milk-free" pellet diet (Harlan Laboratories, Udine, Italy); EHCF (Nutramigen, Mead Johnson Nutrition, Evansville, IN, USA); or EHCF+LGG (Nutramigen LGG 1, Mead Johnson Nutrition, Evansville, IN, USA). The composition of the three experimental diets is presented in Table 1. After 14 days, mice were sensitized orally using a blunt needle on day 0, 7, 14, 21, 28 with 20 mg of beta-lactoglobulin (BLG) (Sigma-Aldrich, Steinheim, Germany) homogenized in PBS (0.2 ml) mixed with 10 µg cholera toxin (CT) as adjuvant. One week after the last sensitization (day 28), acute allergic skin response was assessed and after 24 h, mice were challenged by gavage with BLG to determine anaphylaxis score and rectal temperature. On next day mice were sacrificed, blood samples were collected, and spleens were aseptically excised, lysed and cryopreserved.

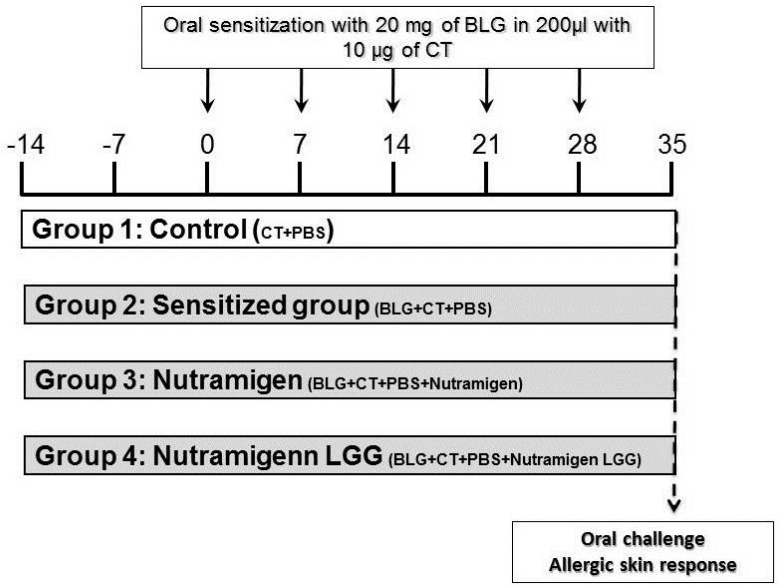

Figure 8 . To explore the effects of EHCF and EHCF plus LGG in CMA prevention

BLG-sensitized mice showed a significant higher acute allergic skin response and anaphylactic symptom score, as compared to control animals. All these effects were significantly reduced by pre-treatment with EHCF or with EHCF+ LGG, but the presence of LGG induced a more pronounced effect on acute allergic response (Figure 9). Similarly, BLG sensitized animals showed a significant increase in anti-BLG IgE serum level, IL-4 production by spleen lysates, and intestinal permeability, compared to control animals. These effects were significantly inhibited by EHCF. The presence of LGG induced a more pronounced effect on all these variables (Figure 9).

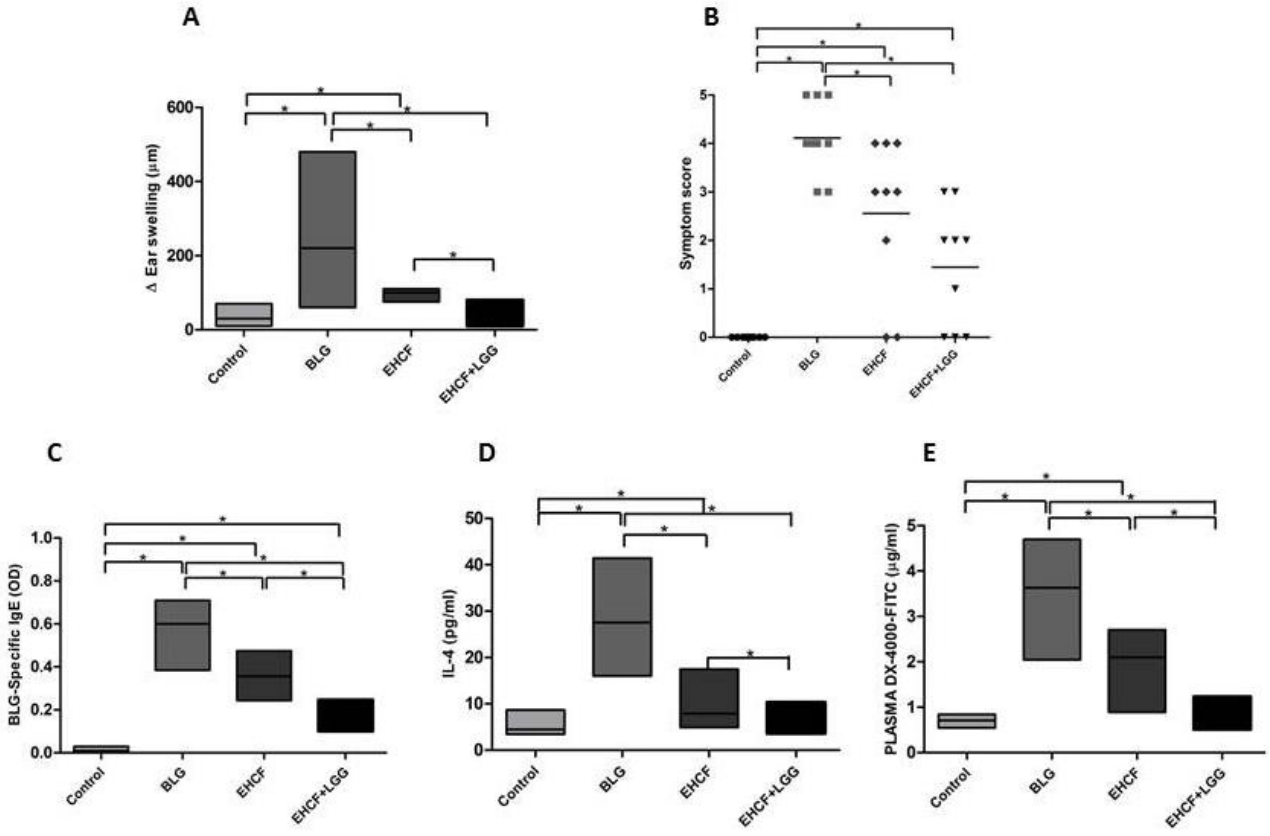

Figure 9 . EHCF and EHCF plus LGG prevent the allergic response to the cow's milk protein β-lactoglobulin (BLG) in a murine model of food allergy (A) acute allergic ear swelling response (B) anaphylactic symptom scores (C) BLG-specific IgE (D) IL-4 from spleen lysates (E) intestinal permeability to plasma DX-4000 FITC. (B), independent samples t-test; (A-C-D-E): Mann-Whitney U Test; *$p<0.01$; **$p<0.05$.

3.4 CMA treatment

The design of the experiments is reported in Figure 10. Briefly, mice were sensitized orally, using a blunt needle on day 0, 7, 14, 21, 28 to 20 mg of BLG homogenized in PBS (0.2 ml) mixed with 10 µg CT as adjuvant. After last sensitization mice were given three different experimental diet: control solid "milk-free" pellet diet; EHCF; or EHCF+LGG. The weight of the mice was monitored weekly. At the end of the 4-week treatment with different diets, acute allergic skin response was assessed and after 24 h mice were challenged by gavage with BLG to determine anaphylaxis score and rectal temperature. On next day mice were sacrificed, blood samples were collected, and spleens were aseptically excised, lysed and cryopreserved.

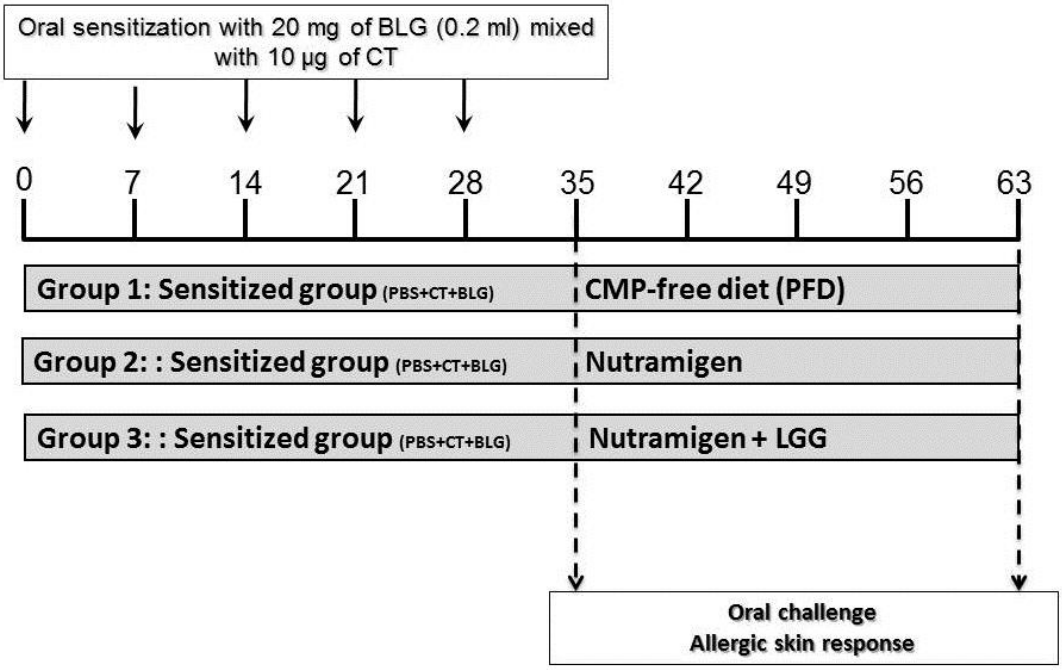

Figure 10 . To explore the effects of EHCF and EHCF plus LGG in the treatment of CMA

Treatment with EHCF was able to significantly reduce acute allergic skin response and anaphylactic symptom score as compared to control animals. The presence of LGG induced a more pronounced effect on acute allergic response (Figure 11). Dietary treatment with EHCF induced a significantly reduction of anti-BLG IgE serum level, IL-4 production by spleen lysates and intestinal permeability increase (Figure11).

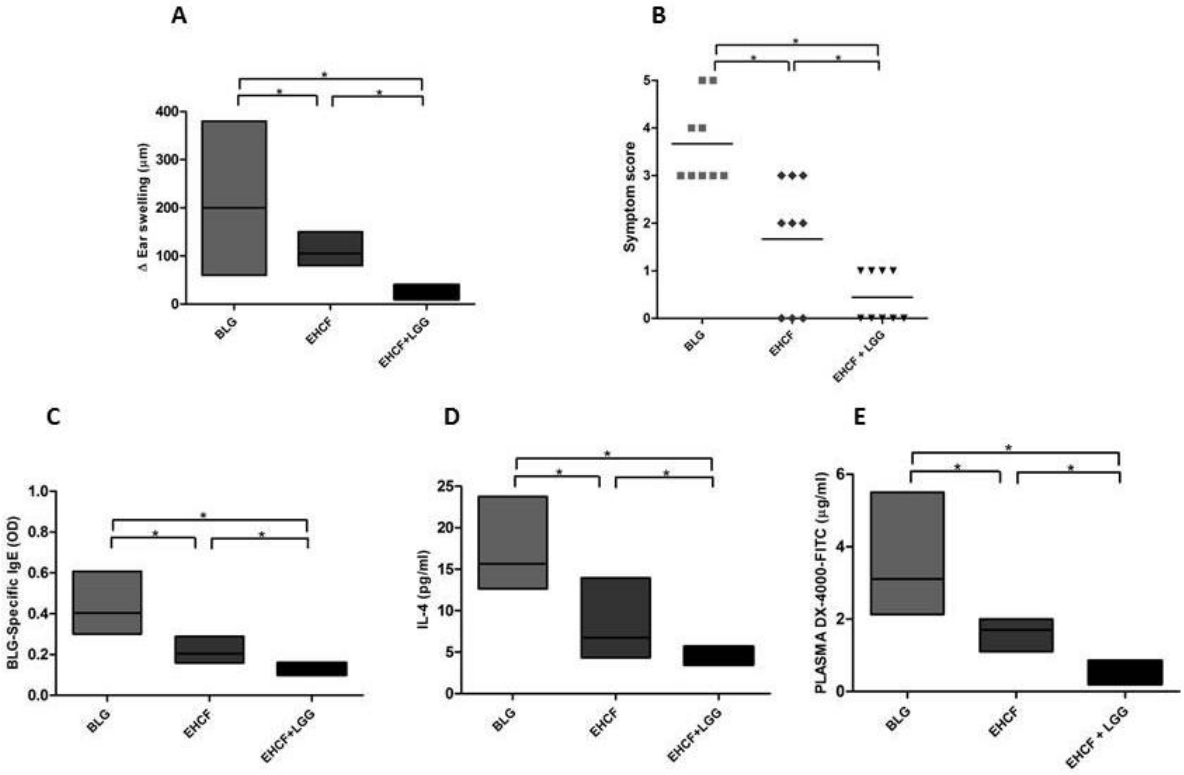

Figure 11 . EHCF and EHCF plus LGG reduce the allergic response to the cow's milk protein β-lactoglobulin (BLG) in a murine model of food allergy (A) acute allergic ear swelling response (B) anaphylactic symptom scores (C) BLG-specific IgE (D) IL-4 from spleen lysates (E) intestinal permeability to plasma DX-4000 FITC. (B), independent samples t-test; (A-C-D-E): Mann-Whitney U Test; *p<0.01; **p<0.05

Conclusive remarks

We obtained an effective oral BLG sensitization in C3H/HeOuJ mice characterized a skin reaction, occurrence of severe allergy-related symptoms, body temperature reduction, associated with an increased IL-4 and anti-BLG specific IgE production. These results resembled what previously obtained by others. (132-133). In addition, a significant increase in intestinal permeability was demonstrated. An increase of gut permeability is a common feature in patient affect by food allergy (Kirsi M. Järvinen, M 2014). Intestinal permeability has been considered a crucial factor in the oral tolerance mechanism. These features were used in our study as biomarkers to explore the effects of a dietary intervention with EHCF alone or in combination with LGG.

Our results show that EHCF, at least in part through a modulation of IL-4 and anti-BLG specific IgE production, is able to prevent oral BLG sensitization. As demonstrated by a significant decrease in acute allergic skin response, anaphylactic symptoms, and body temperature and intestinal permeability modification. These findings are in line with those previously reported in clinical studies demonstrating an allergy preventive effect elicited by extensively hydrolysed casein formulae (134-135). EHCF has been proposed as appropriate alternative to breast milk for allergy prevention in infants at risk. (136-139).

Similar effects were obtained in mice treated with EHCF after sensitization with BLG. In those mice we observed a decrease in IL-4 and anti-BLG specific IgE that lead a reduction in anaphylactic reactions and restoring of intestinal permeability. These findings support the EHCF efficacy previously demonstrated in clinical studies that considered this formulae the first choice to treat patients affect by CMA (140-142). These result was reinforced with

demonstrated that EHCF induces a faster oral tolerance acquisition if compared with other dietary intervention. (118).

All these date provide evidences on a immunoregulatory effect elicited by EHCF that could be due to the presence of immunoregulatory peptides derived from casein hydrolysis. Both suppressive and enhancing effects on immune variables have been found in studies investigating casein-derived peptides (120). These date suggest that the oral ingestion of the casein phosphopeptide preparation may reduce allergic symptoms in animals through suppression of absorption of allergens from the intestinal tract, suppression of IgE formation specific to allergens and/or inhibition of binding of allergens to their IgE on mast cells. One of the best characterized immunomodulatory peptide derived from bovine milk caseins is casein phosphopeptide from β-CN (f1-28) that has been show a mitogenic activity on mouse spleen and rabbit Peyer's patch cells and stimulated their IgA production(143). Moreover, it was able to reduced allergic symptoms mediated by IgE in NC/Jic Jcl mice treated with ovalbumin. The author have also demonstrated that the spleen cells of mice given a peptide-added diet produced lower IL-4 than those of control diet (144).

Recently a β-CN (f 184–202) peptide has been shown to be NFκB inhibitory peptide(123) while the peptide (94-123) of bovine β-casein from yoghurt has been reported to maintain intestinal homoeostasis through production of the secreted mucin MUC2 and of the transmembrane-associated mucin MUC4.(124). Mucus lubricates the intestinal surface, limits passage of luminal molecules into the mucosa, functions as a dynamic defensive barrier against enteric pathogens and acts as a substrate and a niche which the commensal flora can colonize (125) and it could be another of the putative mechanism of the positive effects of EHCF as demonstrated by our result of a protective effect on intestinal permeability.

In the two experimental phases of this study the effect of EHCF was reinforced by the addition of the probiotic Lactobacillus rhamnosus GG. This date further supported clinical evidences showing a more potent activity elicited by EHCF LGG on CMA-related symptoms and gut inflammation [28], and on oral tolerance acquisition which resulted in a higher rate of acquisition of tolerance after 12 months of treatment[7,8]. Probiotics have been reported to modulate immune response and their supplementation has been proposed as a preventive intervention (145). LGG is known to modulate immune functions via various pathways, including those involving enterocytes, monocytes, mast-cells, dendritic cells, and regulatory T cells (146). In particular LGG has been able to suppress Th2 responses such as reduced hypersensitivity scores and lowered serum CMP-specific IgG1 and promote Th1 responses by causing elevated IFN-γ and CMP-specific IgG2a levels in mice that were sensitized with the whole CMP (147). In cell culture of CaCo2 has been demonstrated that LGG is also able to suppress TNF-α-induced NF-κB activation as evidenced by maintenance of epithelial barrier integrity against pro-inflammatory cytokine stimuli and by a reduction in the secretion of specific chemokines induced by activation of this signal transduction pathway (148). Finally, it has been recently demonstrated that daily supplements of LGG resulted in a dramatic shift in the composition of the intestinal microbial community with a large increase in the number of taxa previously associated with a decreased risk for the development of allergy and atopy (149) and taxa with the potential to produce butyrate (150).

We cannot exclude that even components included in the formulae used in the study could lead contributed to immune effects evidences in both experimental phases. Among this the polyunsaturated fatty acids (PUFA) that has been demonstrated are able to reduce the incidence of food allergy and IgE-associated eczema in children at risk for atopy (151). Moreover, dietary supplementation with PUFA largely reduce ovalbumin allergy in mice

(152) and prevent oral sensitization to the cow's milk protein through the induction of functional Treg (153)

In conclusion the result of this study demonstrated an immunoregulatory activity elicited by EHCF that is able to efficiently contour act several mechanisms involving in CMA pathologies. According to previous clinical trials the presence of LGG is able to up-regulate the magnitude of these effects.

Technologies

HPLC Analysis

HPLC of the synthetic peptide was performed using a reverse-phase column (2.0 mm i.d. × 250 mm, C18, 5 μm; Phenomenex, Hesperia, CA) with a flow rate of 0.2 mL/min on an Agilent 1100 modular system with an integrated diode array detector (Palo Alto, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) (v/v) in water; solvent B was 0.1% TFA in acetonitrile. A 60 min gradient of 5–60% buffer B was used. The chromatographic separation of the peptide was performed at ambient (25 °C), low (~4 °C), and high (50 °C) temperatures, using a thermostatic column holder or chilling the column in an ice-cold bath.

Cell culture and treatments

Caco-2 cells were cultivated in Dulbecco Modified Eagle's Medium DMEM, (GIBCO) 10% FCS (GIBCO), 100 units/ml penicillin-streptomycin (GIBCO), 1 mM glutamine. LPS-free P31-43 and its synthetic mutants (Inbios, .95% purity, MALDI-toff analysis as expected) were obtained by Ultrasart-D20 (Sartorius AG, Goettingen, Germany) filtration. LPS levels were below detection (,0.20 EU/mg), assessed by commercial QCL-1000 kit (Cambrex Corporation, New Jersey USA). P31–43 sequence: LGQQQPFPPQQPY and its ala mutants were used at 500 mg/ml and the caco2 cells were stimulated for 30min.

Immunoprecipitation and immunoblotting

Cell lysates were prepared as described previously(154) and the phosphorylated form of Erk [p-Erk] was detected using anti-p-Erk E-4 mouse monoclonal antibody (Clone E4 Santa Cruz). Total Erk was detected using Erk rabbit polyclonal antibody anti-Erk K23 (clone K23, Santa.Cruz).

Briefly, 2 micrograms of anti p-Erk E-4 immunoprecipitating mouse monoclonal antibody (clone 528,Santa Cruz) were added to 1 milligram of cell lysates in 500 micro litres of lysis buffer kept for 2 h at 4°C with gently rocking. Thirty micro litres of 1:1 protein-A agarose (Pierce) was added and the mixture was rocked gently for 1 h at 4°C, before centrifugation at 12 000 g for 5 min at 4°C. The immunoprecipitate was washed three times with 500 ml of lysis buffer. Proteins were separated by SDS-PAGE and immunoblotted with anti-phosphotyrosine (p-Tyr) mouse monoclonal antibody (clone 4G10 from UBI) or anti-EGFR rabbit polyclonal antibody (clone 1005, Santa Cruz). Electrophoresis and immunoblotting

were all performed as described elsewhere (154). Densitometric analysis was performed as follows. Band intensity was evaluated by integrating all the pixels of the band without the background, calculated as the average of the pixels surrounding the band. Phosphorylation increments (Pi) were calculated from the ratio of phosphorylated (P) and unphosphorylated (UP) bands in treated (t) and untreated cells (ut) as follows: Pi=(Pt/UPt)/(Put/UPut).

Data bank analysis

Swissprot, Trembl and InterPro data banks were searched for sequences matching peptide P31–43, by using Blast and FastA. Sequence alignment was performed by using ClustalW and visualised by PrettyPlot from the EMBOSS suite.

Statistical analysis

Statistical analysis was performed where appropriate by employing Student's t-Test; asterisks mark results whereP,0.05.

Acute allergic skin response, anaphylaxis symptom score and body temperature

Acute allergic skin response was evaluated by ear thickness measurement determined in duplicate using a digital micrometer (Mitutoyo, Lainate, Milano) 1 hour after intradermal injection of 0.5μg of BLG in the ear pinnae. The ear swelling was calculated by correcting the allergen-induced ear thickness with the basal ear thickness. The delta ear swelling was expressed as μm.

Within one hour following the oral BLG challenge (50 mg in PBS by gavage), anaphylaxis was assessed by measuring changes in body temperature and recording symptoms score. Rectal temperature was measured before and 60 min after oral challenge. Hypersensitivity symptoms were scored by an investigator blind to the study group assignment, as previously described (121): 0 = no symptom; 1 = scratching and rubbing around the nose and head; 2 = reduced activity; 3 = activity after prodding and puffiness around the eyes and mouth; 4 = no activity after prodding, labored respiration, and cyanosis around the mouth and the tail; and 5 = death.

Serum BLG-specific IgE

Blood samples obtained by intracardiac puncture from mice were collected into serum separator tubes. The serum portion was separated by centrifugation at 10,000 x g for 5 min

at 20 ºC. Serum samples were then aliquoted into Eppendorf tubes and stored at -20 ºC until being analyzed. BLG-specific serum IgE were detected by ELISA. Briefly, 96-well plates were coated with 100 μg/mL of BLG in 0.1 mol/L Na-bicarbonate/carbonate coating buffer (pH 9.6). After overnight incubation at 4 °C, plates were washed 3 times with 150 μl of PBS plus 0.05% Tween-20 (PBS-T) and blocked with 100 μl of 2% BSA in PBS-T for 2 h at 37 °C. Subsequently, the plates were washed 3 times and 100 μl of serially undiluted serum samples were added to the wells and incubated at 37 °C for 90 min. Plates were then washed 3 times, and 100 μL of horseradish peroxidase (HRP) conjugated anti-mouse antibodies were added to each well. The plates were again incubated at 37°C for another 60 min and washed 3 times. Then, 100 μl of TBS were added to each well and 15 min were allowed for the development of colorimetric reactions. Absorbance were read at a wavelength of 450 nm in a microplate reader.

Interleukin 4 in spleen lysates

Spleen lysates were obtained as previously described (155). Briefly, individual spleens were placed in Eppendorf tubes containing 0.5 ml of lysate buffer. The spleen cells were lysed and homogenized by sonication for 30 s on ice. Supernatants were collected after centrifugation at 17,500 g for 10 min at 4 ºC and stored at -20ºC. Interleukin-4 (IL-4) from spleen lysates were analyzed by commercially available ELISA kits following the manufacturer's protocol.

Intestinal permeability in vivo

From each study group of the two experimental phases (prevention and treatment) three animals were used to investigate intestinal permeability, as previously described (156). Briefly, mice were fasted for 6 h and then gavaged with 4,000 Da FITC-labeled dextran diluted in water (TdB Consultancy AB, Uppsala, Sweden) (500 mg/kg, 125 mg/ml). After 2 h, blood (500 μl) was collected from intracardiac puncture and centrifuged (3,000 rpm for 15 min at RT), and FITC-dextran concentration in plasma was determined by spectrophotometry (excitation wavelength 485 nm; emission wavelength 535 nm; HTS-7000 Plus-plate-reader; Perkin Elmer, Wellesley, Massachusetts, USA),

Statistical analysis

For categorical variables, the $\chi 2$ test and Fisher's exact test were used. Results were reported as means and 95% confidence interval (CI) and as median and interquartile range

(minimum-maximum) due to non-parametric distribution (established by the Kolmogorov test). The level of significance for all statistical tests was 2-sided, $p<0.05$. SPSS 16.0 for Windows software (SPSS Inc, Chicago, Ill., USA) was used for data analyses.

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