Chapter 7

New Tools for the Diagnosis of Celiac Disease

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Doi: http://dx.doi.org/10.3926/oms.255

How to cite this chapter

Fernández-Bañares F, Farré C, Carrasco A, Mariné M, Esteve M. *New Tools for the Diagnosis of Celiac Disease.* In Arranz E, Fernández-Bañares F, Rosell CM, Rodrigo L, Peña AS, editors. *Advances in the Understanding of Gluten Related Pathology and the Evolution of Gluten-Free Foods.* Barcelona, Spain: OmniaScience; 2015. p. 259-276.

Abstract

New tools for celiac disease (CD) diagnosis may be of help in at least three frequent clinical situations: 1) HLA-DQ2/8+ individuals on a self-prescribed gluten-free diet; 2) Patients with seronegative villous atrophy; and 3) HLA-DQ2/8+ patients with lymphocytic enteritis and either positive (often with low/borderline titers increasing the risk of false positives) or negative celiac serology.

The $\gamma\delta$+ IEL count, assessed by either immunohistochemistry or flow cytometry, may help to identify CD patients when serology and clinical data are not conclusive, or when the histological diagnosis remains equivocal. The detection of subepithelial tissue transglutaminase antibodies seems to be very sensitive and specific in diagnosing CD in patients with potential CD or seronegative villous atrophy. The presence of these autoantibodies reinforces the CD diagnosis in borderline cases. EmA or anti-tTG2 assay of the culture medium of intestinal biopsy specimens in patients with negative serology, but with symptoms suggestive of CD and the HLA-DQ2 and/or HLA-DQ8+, seems to be a good option to help confirm the diagnosis of CD. It also may be useful in suspected cases showing conflicting laboratory and histological data. The whole blood cytokine release assays (ELISPOT) seems to be both sensitive and specific for detection of gluten-reactive T cells in CD; further clinical studies addressing the utility of these tests in patients with an uncertain diagnosis of CD is warranted. The tetramer test may be of help to confirm the diagnosis of CD after a short 3-days gluten challenge. However, the results seem comparable to the ELISPOT test; for that

reason, and also taking into account that the tetramer test is technically difficult, widespread use of the test is almost not expected.

Keywords

Celiac disease, 'celiac-lite' disease, potential celiac disease, $\gamma\delta$+ cells, subepithelial tissue transglutaminase antibodies, tTG2 in culture of intestinal biopsy, ELISPOT test, tetramer test.

1. Introduction

Celiac disease (CD) is an enteropathy caused by an immune reaction triggered by dietary gluten, a protein found in wheat, rye, barley, and some varieties of oats, which manifests in genetically predisposed individuals. Since the first morphological lesion description by John Paulley in 1954, CD diagnosis was based precisely on the demonstration of the characteristic, gluten-dependent small intestinal lesion. This basic general concept is still valid. However, in recent decades, the discovery of accurate diagnostic methods (serological and genetic), through mass screening techniques or evaluating at-risk groups, has allowed the identification of large numbers of patients with silent or paucisymptomatic forms. This has afforded the knowledge that CD is not a rare disease, that its spectrum of clinical manifestations, both in type and severity, is very wide, and that there is not always a correlation between the severity of the histological lesion and intensity of the clinical manifestations. In this regard, an important change in CD diagnostic criteria has been the gradual acceptance that histological mild enteropathy forms (type 1 Marsh lesions, also called lymphocytic enteritis, lymphocytic enteropathy or lymphocytic duodenosis) are also part of the CD spectrum and must to be treated as such, when they produce clinically relevant symptoms or signs[1].

Tissue transglutaminase IgA class autoantibodies (anti-tTG2) are the serological markers of choice for the detection of CD as recommended by the ESPGHAN. The anti-tTG2 are equivalent to the classic endomysial IgA autoantibodies (EmA). After the identification of transglutaminase as the autoantigen by itself, anti-tTG2 are determined by a quantitative and automated immunoassay, overcoming the technical drawbacks of indirect immunofluorescence used to determine EmA. This remains a manual, subjective and qualitative technique. The recommendations on how, when and to whom perform serum anti-tTG2 have been recently reviewed[2].

It is well known that celiac serology may be negative in the milder forms of CD[2]. In this context, gluten challenge has been performed in order to

determine if it worsens the histological lesion or if antibodies become positive, which would lead to CD diagnosis[3,4]. Though, this requires repeated endoscopies, before and after gluten challenge, that together with symptom relapse are often intolerable for patients, precluding achieving a definite diagnosis.

In addition, the overlap between patients with non-celiac gluten sensitivity and celiac disease patients with type I Marsh lesion becomes evident and differential diagnosis quite difficult often clinicians are confronted with the challenge of patients who choose to live without gluten, even without a proper diagnosis of CD. This is particularly so as both the serology and small intestine histology normalize in CD patients on a gluten-free diet. In those circumstances, HLA genotyping is of value, since CD is extremely improbable in those patients who are HLA-DQ2/8 negative, but it is not enough in HLA-DQ2/8 positive patients, since 30-40% of the healthy population are also positive.

Thus, new tools for CD diagnosis may be of help in at least three frequent clinical situations: 1) HLA-DQ2/8+ individuals on a self-prescribed gluten-free diet; 2) Patients with seronegative villous atrophy; and 3) HLA-DQ2/8+ patients with lymphocytic enteritis and either positive (often with low/borderline titers increasing the risk of false positives) or negative celiac serology. Also it would be interesting for monitoring gluten reactivity in latent or potential CD, as well as in first-degree relatives with the highest risk of developing the disease.

2. When Does Celiac Serology Fail in the Diagnosis of Celiac Disease?

It is well known that celiac serology is often negative in the milder forms of CD: in 30% of the patients with partial villous atrophy and up to 80% of those with Marsh 1 lesions[5]. Since histological damage is worse at clinical presentation in children than in adults[6], seronegative CD is more frequent in adult patients.

Prospective studies have shown that the diagnostic accuracy of serology is not as high as described, since there is around 10-20% seronegative CD patients[7,8]. We agree with the opinion of Catassi and Fasano who affirmed that 'Seronegative CD is likely to be underestimated due to the tendency to perform small-intestinal biopsy only in patients with positive-CD serum markers (so-called self-fulfilling prophecy)[9].

Although there are other etiologies of villous atrophy, it is important to take in mind that the most frequent etiology of villous atrophy in a patient with negative CD serology is CD[10]. Finally, we should not misinterpret as negative the IgA serology results obtained in patients with IgA deficiency, in children under two years of age, in patients on immunosuppressive treatment, or in patients on a gluten-poor or gluten-free diet since a few weeks without gluten can give a negative serological result.

False positive anti-tTG results have been described in adult patients with autoimmune diseases[11], acute coronary disease[12], primary biliary cirrhosis[13], psoriasis[14], chronic inflamed ileal pouches[15], and children with common infections[16]. Low titers or borderline values are more often associated to false positive results.

3. Usefulness of Intraepithelial $\gamma\delta$+ Determination

The TCR$\gamma\delta$+ intraepithelial lymphocyte (IEL) determination is considered useful in doubtful or difficult CD cases[17]. In CD patients these $\gamma\delta$+ T cells are increased in all stages of the disease, both in untreated CD and under the gluten-free diet[17]. It has also been observed that they are increased both in potential and latent CD[18,19]. The $\gamma\delta$ IEL increase is not totally specific to CD, since it has occasionally been found in other conditions such as cow's milk intolerance, food allergy, cryptosporidiosis, giardiasis, Sjögren syndrome, and IgA deficiency[17]. However, the increase in $\gamma\delta$ IEL in a minority of patients with these conditions tends to be mild and transient[17]. It has been stated that CD is the only disease in which $\gamma\delta$ IEL, are increased systematically, permanently, and intensely[17,20-22].

Assessment of the density of $\gamma\delta$ IEL is in general performed with immunohistochemistry techniques. Noteworthy, Järvinen et al. reported that $\gamma\delta$+ T cells had a positive predictive value of 95% and a negative predictive value of 85%, in the detection of CD[23]. An increase in this type of cells has also been detected in most patients with CD mild enteropathy[24]. Identification and count of $\gamma\delta$+ T cells are usually performed on cryosectioned snap-frozen biopsy, which have limited its use to the research setting and has rarely been adopted for routine clinical practice. Recently, a new anti-TCRγ antibody, suitable on formalin-fixed paraffin-embedded samples, has been described, and its feasibility to count $\gamma\delta$+ T cells together with CD3 cells in patients with lymphocytic enteritis has been demonstrated[25].

Lymphogram on IEL isolated by flow cytometry has been proposed, as an initial screening for CD. Using this technique, an IEL pattern typical of CD (CD IEL cytometric pattern) was defined, consisting of both an increase in $\gamma\delta$+ IEL and a decrease in CD3- IEL (reviewed by Leon F)[17]. The concomitant decrease in CD3- IEL provides increased specificity for the diagnosis of CD[26]. A description of this CD3- IEL population has been made, showing a CD3- CD7+ CD103+ CD45+ phenotype[18,26,27].

Flow cytometry is a powerful analytical tool for the study of IEL, compared to immunohistochemistry. It allows the analysis of a greater number of cells and yields a computerized record of the results. It gives fast, sensitive, reproducible and objective semi-quantitative results. Since an increase of CD3+TCR$\gamma\delta$+ and a decrease in CD3- IEL has been previously described as a characteristic flow cytometric pattern of CD with atrophy[17,18,28], a recent study[29] assessed the usefulness of this technique for diagnosing lymphocytic enteritis due to CD. In this study, 205 patients who underwent duodenal biopsy for clinical suspicion of CD and positive HLA-DQ2 and/or HLA-DQ8 were evaluated. Fifty patients had villous atrophy, 70 patients lymphocytic enteritis, and 85 had normal histology. Duodenal biopsies were obtained to assess two typical flow cytometric patterns: complete CD flow cytometric pattern was defined when TCR$\gamma\delta$+ was increased and CD3- decreased, and incomplete CD flow cytometric pattern was defined when an isolated TCR$\gamma\delta$+

increase was detected. Anti-TG2 IgA subepithelial deposits were also assessed. Sensitivity of anti-TG2 intestinal deposits, and complete and incomplete cytometric patterns for CD diagnosis in patients with positive serology (Marsh 1+3) was 92%, 85% and 97% respectively, but only the complete cytometric pattern had 100% specificity. Taking into account these definitions and the response to a gluten-free diet, we studied HLA-DQ2/8+ patients with lymphocytic enteritis and negative serology to either confirm or ruled out CD. CD cytometric pattern showed a better diagnostic performance than anti-TG2 intestinal deposits to detect CD in the initial diagnostic biopsy of these patients. This methodology allowed to establish the diagnosis of CD in more than twice the number of patients with lymphocytic enteritis diagnosed on the basis of serological results alone.

In conclusion, the $\gamma\delta$+ IEL count, assessed by either immunohistochemistry or flow cytometry, may help to identify CD patients when serology and clinical data are not conclusive, or when the histological diagnosis remains equivocal.

4. Diagnostic Utility of Tissue IgA Transglutaminase Subepithelial Deposits.

It has been shown that the production of CD autoantibodies, takes place locally in the small intestinal mucosa, and subsequently circulate into to the bloodstream. However, besides being detectable in the bloodstream, these autoantibodies remain sequestered in the place where they have been produced. In untreated CD it is possible to detect IgA tTG deposits in the intestinal mucosa subepithelially and around blood vessels of the lamina propria[30]. Interestingly, these deposits can be detected in patients with positive EmA and without villous atrophy[24, 30-32] and even in patients with negative serology and Marsh type 1 to 3 lesions[33-35]. In a recent study on untreated CD patients, it was demonstrated that 100% of 261 patients with villous atrophy had subepithelial IgA tTG deposits (9% had negative serum EmA), 90% had moderate to strong intensity. In contrast, 18% of the controls

had deposits of minor intensity. After a gluten-free diet, there was a gradual decrease in the intensity of these deposits, which remained positive, in the long term, in 56% of the patients. The sensitivity and specificity of these deposits for CD diagnosis was of 100% and 82%; however, serology sensitivity and specificity were of 91% and 100%, respectively[35]. In a study on children with positive EmA or tTG and positive genetics (HLA-DQ2 or DQ8) but without villous atrophy, IgA tTG deposits were detected in 85% of 39 patients. Similarly, a study on another group of children revealed negative serology and Marsh type I lesions, with increased TCR$\gamma\delta$+ intraepithelial lymphocytes, allowing the detection of IgA tTG deposits in 66% of 18 patients. These deposits were detected in 9% of 34 children with normal intestinal mucosa and absence of gluten sensitivity markers[34]. Another recent study showed that IgA tTG deposits were detected in 12 of 20 (60%) adult patients with Marsh type I lesions diagnosed with CD on the basis of the "4 of 5" rule by Catassi and Fasano[9]; four of these 12 positive patients were seronegative[29].

In conclusion, the detection of subepithelial tissue transglutaminase antibodies seems to be very sensitive and specific in diagnosing CD in patients with potential CD or seronegative villous atrophy. The presence of these autoantibodies reinforces the CD diagnosis in borderline cases.

5. Anti-tTG2 and EmA Assays in the Culture Medium of Biopsy Samples

The assay of the culture medium of intestinal biopsy specimens for EmA or anti-tTG2 antibodies can help to identify as CD either the infiltrative/hyperplastic (Marsh 1-2) or the partial villous atrophy (Marsh 3a) lesions often associated with negative serology[8,36,37]. In a study[37], EmA and anti-tTG assayed in the culture medium had 98% sensitivity, 100% specificity, and 98% diagnostic accuracy. These assays were positive in 24 out of 29 seronegative CD patients (77% with partial villous atrophy, and 23% with lymphocytic enteritis). In another study by the same group[8], EmA assay in

the culture medium had a higher sensitivity (98 vs. 80%) and specificity (99 vs. 95%) than serum EmA and/or anti-tTG assay. In that study, 32 adults and 39 children had a seronegative CD (17% of 418 CD patients).

In addition, combined serum and supernatants of cultured intestinal duodenal biopsy anti-tTG assessment increased CD serological sensitivity from 19% to 30% in Marsh I patients carrying the risk haplotypes HLA-DQ2 and/or HLA-DQ8[38]. It was concluded that supernatants of duodenal biopsies anti-tTG detection improves serological determination sensitivity in Marsh I patients, providing diagnostic value and therapeutic impact.

The diagnostic yield of the anti-tTG2 assay of the culture medium of biopsy seems to be similar, or perhaps better, that the diagnostic accuracy of IgA tTG subepithelial deposits. However, a recent study comparing the two techniques suggests that the measurement of antibodies secreted into culture supernatant is the best method for detecting intestinal anti-tTG2 antibodies[39].

In conclusion, EmA or anti-tTG2 assay of the culture medium of intestinal biopsy specimens in patients with negative serology, but with symptoms suggestive of CD and the HLA-DQ2 and/or HLA-DQ8+, seems to be a good option to help confirm the diagnosis of CD. It also may be useful in suspected cases showing conflicting laboratory and histological data.

6. IFN-γ ELISPOT

The histological features of the small intestine of celiac disease probably result from an increased Th1-deviated immune response. Gluten appears to induce a non-proliferative activation of CD4+ lamina propria T-cells, especially activated Th1-like cells secreting IFN-gamma[40]. However, one year after the introduction of a gluten-free diet, the transcription of IFN-gamma is downregulated[41].

Enzyme Linked Immuno-spot (ELISPOT) is a technique by which immune markers, e.g., cytokine and chemokine secretion, can be detected at the single-cell level, since secreted cytokines are captured and accumulated in the ELISPOT plate[42].

In children with untreated CD, the number of IFN-gamma-producing cells, detected by ELISPOT, is shown to be increased and actually, after gluten challenge, the numbers of IFN-gamma-producing cells still remain high[43].

It has also been shown that an *in vivo* gluten challenge is a simple and safe method that allows gliadin-specific T-cells to be analyzed and quantified in peripheral blood by ELISPOT[44]. This technique could differentiate patients with CD from other patients who have adopted a gluten-free diet. No T cell assay could distinguish between CD patients and controls prior to gluten challenge, but after gluten challenge the IFN-γ ELISPOT was 85% sensitive and 100% specific for CD patients[45].

As an added benefit over current diagnostic tests being performed on patients already following a gluten-free diet, the mobilization of gluten-reactive T cells specific for CD into the bloodstream requires oral gluten challenge for only 3 days, instead of the weeks or months required for diagnosis based on abnormal small bowel histology. Oral gluten challenge consists of four slices (4 x 50 g) of white bread daily for three days[44]. Blood for cytokine release assays is drawn immediately before and on day 6 after starting with the gluten challenge, or prior to begin a gluten-free diet in untreated CD patients.

In conclusion, the whole blood cytokine release assays seems to be both sensitive and specific for detection of gluten-reactive T cells in CD; further clinical studies addressing the utility of these tests in patients with an uncertain diagnosis of CD is warranted.

7. HLA-DQ2-Gliadin Tetramer Assay

Brottveit et al. recently assessed the potential of a fluorescence-activated cell sorter (FACS)-based assay utilizing MHC class II-peptide tetramers detecting DQ2 · 5-glia-α1a and DQ2 · 5-glia-α2 epitope-specific T cells in blood, after 3-days gluten challenge, for the diagnosis of CD in patients following a gluten-free diet[46]. This tetramer assay was 85% sensitive and 100%

specific for HLA-DQ2.5+ CD[46]. Recently, these findings using MHC tetramers have also been replicated in CD patients from the United States[47].

This test, as the ELISPOT assay, may be a superior method to diagnose CD in individuals currently on a gluten-free diet. Available tests, including antibody levels and intestinal biopsy results, can be completely normal in CD patients on a gluten-free diet. These individuals are often asked to reintroduce gluten-containing foods for 2-4 weeks prior re-testing for an accurate diagnosis. This clinical practice may be intolerable in some patients precluding the definite diagnosis. In contrast, a short-term gluten exposure is, in general, well tolerated.

In conclusion, the tetramer test may be of help to confirm the diagnosis of CD after a short 3-days gluten challenge. However, the results seem comparable to the ELISPOT test; for that reason, and also taking into account that the tetramer test is technically difficult, quite laborious and the tetramer reagents have limited stability, widespread use of the test is almost not expected.

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