Journal of Pediatric Gastroenterology and Nutrition
43:613–618 © November 2006 Lippincott Williams & Wilkins, Philadelphia

A New Indirect Chemiluminescent Immunoassay to Measure Anti–tissue Transglutaminase Antibodies

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ABSTRACT

Objectives: Anti–tissue transglutaminase antibody (anti-tTG) determination using second-generation (human antigen) enzyme-linked immunoassays (ELISAs) is a very accurate test to diagnose celiac disease (CD). In this study, we compared 2 second-generation ELISAs (Celikey tTG; Pharmacia Diagnostics GmbH & Co, Freiburg, Germany, and QuantaLite; Inova Diagnostics, San Diego, CA) and antiendomysial antibodies (EMAs) with a new indirect chemiluminescence immunoassay (LIAISON tTG; DiaSorin S.p.A., Saluggia, Italy) in diagnosing and monitoring CD in children.

Patients and Methods: Antiendomysial antibodies, anti-tTGs and total immunoglobulin A were measured in the sera of 103 control children, 101 children with histologically proven CD and 31 CD children on gluten-free diet (GFD).

Results: Anti–tissue transglutaminase antibody mean levels were significantly higher in CD with respect to control or GFD children. The sensitivity value of EMAs, LIAISON tTG, Celikey tTG and QuantaLite in diagnosing CD was 97.7%, 97.0%, 94.1% and 98.0%, respectively, and the corresponding specificity values were 91.1%, 98.1%, 97.1% and 96.1%, respectively. The degree of mucosal destruction (Marsh criteria) was correlated with EMA semiquantification ($P < 0.01$) and with the circulating levels of anti-tTGs measured using LIAISON ($P < 0.05$) or QuantaLite ($P < 0.01$). Twenty-six CD children were followed up from 5 to 25 months after GFD. The circulating levels of anti-tTGs measured with any of the 3 assays significantly dropped after GFD.

Conclusions: Anti–tissue transglutaminase antibody determination with second-generation ELISAs is as effective as EMAs for CD diagnosis. The novel chemiluminescent method described in the present paper for the detection of anti-tTGs in the diagnosis of CD had the highest sensitivity and specificity values. The anti-tTG test correlates with the degree of mucosal destruction and is suitable for verifying patient compliance to dietary treatment. ***JPGN 43:613–618, 2006.*** **Key Words:** Celiac disease—Anti–tissue transglutaminase—Gluten-free diet—Chemiluminescence—ELISA.

INTRODUCTION

The serological screening of celiac disease (CD) is based on the serum determination of antibodies elicited against reticulin, gliadin, endomysium and tissue transglutaminase (1–13). Antireticulin antibodies is a specific but not sensitive test; antigliadin immunoglobulin (Ig) G or IgA have limitations both in sensitivity and specificity, which usually are less than 80% (14). Antiendomysial antibodies (EMAs) of the IgA class have long been considered the most sensitive and specific (>95%) serological tool for the diagnosis of CD (1–3,14). As this diagnostic approach is based on indirect immunofluorescence, using the esophagus of monkeys or umbilical cord as substrate, it is labor intensive and operator dependent. After the discovery made in 1997 by Dieterich et al. (4) that the antigen recognized by EMA is tissue transglutaminase, now known as TG2 (15), several enzyme-linked immunoassays (ELISAs) have been realized to determine the anti-TG2 antibody. The advantages of ELISA assays over indirect immunofluorescence are as follows: are not operator dependent, can be automated and do not use material from protected animal species. They have therefore been rapidly introduced into clinical laboratories.

Enzyme-linked immunoassays for anti-TG2 determination can be subdivided into 2 main categories: first- and second-generation ELISAs (16,17). The former uses guinea pig liver as the TG2 antigen source, whereas the latter use human recombinant or human purified transglutaminase. The pooled estimates of the sensitivity and specificity of the first-generation ELISAs are 93% (95% CI, 88.8–95.9) and 96% (95% CI, 93.1–98.0),

Received March 30, 2005; accepted July 25, 2006.

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respectively (16). One of the main limitations of first-generation assays is their low specificity in patients with autoimmune liver diseases, such as autoimmune hepatitis or primary biliary cirrhosis (18,19). This phenomenon may depend on impurities in the guinea pig liver antigen causing an aspecific reaction. This limitation has been overcome by the second-generation ELISAs, which have sensitivity and specificity values ranging from 91% to 97% (17,20).

Although very sensitive, the ELISA determination of anti–tissue transglutaminase antibodies (anti-tTGs) is not infallible, false-negative results being recorded in some patients with CD. When false negatives are due to IgA deficiency, a reliable finding may be made by determining IgG class anti-tTGs (13,21,22) and, in the remaining false-negative cases not due to total IgA deficiency, the sensitivity of the test may be optimized by enhancing the analytical sensitivity by using chemiluminescent enzyme immunoassays rather than colorimetric ELISAs, as suggested for the determination of other analytes (23–25). Chemiluminescence is emission of light produced during a chemical reaction, and isoluminol and acridinium esters are the most important chemiluminescent labels to be used in this type of immunoassay. In flash chemiluminescence, the chemiluminescent labels, acridinium esters, are bound to one of the components of the immunometric reaction. Its oxidation by alkaline hydrogen peroxide in the presence of a detergent produces a rapid flash of light (26).

The aim of the present study was to verify the analytical and clinical accuracy of a chemiluminescent method in the detection of IgA class anti-tTGs in the diagnosis and follow-up of CD in children and compare this method with 2 commercially available ELISAs and with the use of EMAs.

PATIENTS AND METHODS

Our series consisted of 235 children, 204 of whom consecutively underwent gastrointestinal endoscopy for upper gastrointestinal symptoms and suspected malabsorption at the Department of Pediatrics of the University of Padova. Celiac disease was diagnosed in 101 children (30 boys, 71 girls; age range, 1–15 years) on the basis of histological findings and ruled out in the remaining 103 children (control subjects [CS], 48 boys, 55 girls; age range, 1–16 years). The remaining 31 patients (13 boys, 18 girls; age range, 3–13 years) were on a gluten-free diet (GFD) and had a well-established diagnosis of CD, the diagnosis being made after the criteria defined by the European Society of Paediatric Gastroenterology and Nutrition (27). From 64 of 101 CD children, Marsh classification of celiac lesions was available (28). Among these 64 children, 34 had Marsh type I (infiltrative), 6 had Marsh type II (hyperplastic) and 24 had Marsh type III (destructive) lesions. After diagnosis, 26 of 101 children with CD had a follow-up ranging from 5 to 25 months (median, 14.5 months), being treated with a GFD.

After the parents gave their informed consent, serum samples were obtained in all cases immediately before endoscopy and stored at -20°C for no more than 3 years, until biochemical analysis of EMAs IgA, total IgA and anti-tTG IgA. Antiendomysial antibodies were measured by indirect immunofluorescence using monkey esophagus as substrate (Euroimmun, Lubeck, Germany). A reticular pattern of immunofluorescence observed in the muscularis mucosae with a serum dilution of more than 1:5 was considered positive. The intensity of fluorescence was reported in a semiquantitative way (negative, 1+, 2+, 3+). Because of the limited availability of sera for some children, EMAs were available in 56 CS, 86 CD and 26 GFD.

Total IgA were measured by means of an immunonephelometric assay (Dade Behring GmbH, Liederbach, Germany). Anti–tissue transglutaminase antibodies were assayed using 3 different commercial kits: LIAISON tTG (DiaSorin S.p.A., Saluggia, Italy), Celikey tTG (Pharmacia Diagnostics GmbH & Co, Freiburg, Germany) and QuantaLite (Inova Diagnostics, San Diego, CA). The former was an indirect chemiluminescent immunoassay, whereas the latter 2 were ELISAs. The LIAISON tTG assay uses a label derived from isoluminol, *N*-(4-aminobutyl)-*N*-isoluminol. The human tissue transglutaminase antigens were recombinant from baculovirus for LIAISON tTG and Celikey tTG assays, whereas they were purified from human erythrocytes for QuantaLite. The assays were carried out on automated instruments: LIASON for LIAISON tTG, Minilyser Tecan (Menarini, Italy) for Celikey tTG and QuantaLite. LIAISON tTG within run CVs were assessed by the measurement of 15 aliquots from 3 different samples with low, medium and high values, in one run. Between run CVs were assessed by the measurement of 15 aliquots from 3 different samples with low, medium and high values, in different runs. They ranged from 3.7% to 8.8%.

The statistical analysis of data was made using the following: 1-way analysis of variance, Bonferroni test for pairwise comparisons, Student t test for paired data, the χ^2 test, Spearman correlation analysis and receiver operating characteristic (ROC) curves (SPSS statistical software, version 9.0; Chicago, IL). From ROC curves, we identified the cutoff values associated with the highest sensitivity and specificity by calculating the differential positive rate (DPR) for each cutoff value from the formula DPR = sensitivity − (1 − specificity). The cutoff value with the highest DPR is also the cutoff value associated with the highest sensitivity and specificity.

RESULTS

Figure 1 shows the individual anti-tTG values obtained with the 3 immunoassays tested. At all assays, the mean anti-tTG values were significantly higher in CD than in CS and GFD patients (1-way analysis of variance, $F = 88.2$, $P < 0.001$ for LIAISON tTG; $F = 134.9$, $P < 0.001$ for Celikey tTG and $F = 290.5$, $P < 0.001$ for QuantaLite).

Antiendomysial antibodies, negative in 51 of 56 CS (specificity = 91.1%), were positive in 84 of 86 CD children (sensitivity = 97.7%). Antiendomysial antibodies were negative in 23 of 26 GFD patients; in this

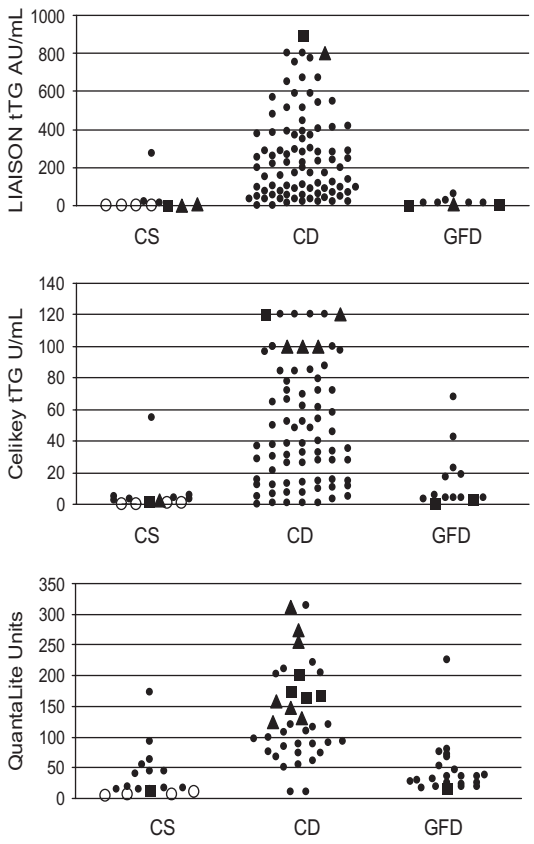

FIG. 1. Individual values of anti-tTG assayed with LIAISON tTG, Celikey tTG and QuantaLite methods. ● = 1 child, ▲ = 5 children, ■ = 10 children, ○ = 20 children.

former group, the 3 cases with positive findings were scored as 1+.

The results of the ROC curve analyses obtained with the 3 anti-tTG assays for comparison between CD and CS are reported in Table 1. The cutoff values corresponding to the highest sensitivity and specificity obtained calculating the differential positive rate are also shown.

Table 2 reports the individual false-negative and false-positive results. In the table, total IgA values are also shown.

Figure 2 shows mean values, SDs and a statistical analysis of anti-tTG results obtained after subdividing CD and GFD children on the basis of EMAs semiquantification. Anti-tTG levels, measured using all 3 investigated methods, were significantly correlated with EMAs semiquantification ($F = 17.17$, $P < 0.001$ for LIAISON tTG; $F = 23.70$, $P < 0.001$ for Celikey tTG and $F = 54.93$, $P < 0.001$ for QuantaLite).

In CD children, the degree of mucosal destruction classified after Marsh criteria was correlated not only with EMA semiquantification ($\chi^2 = 18.6$, $P < 0.01$) but also with the circulating levels of anti-tTGs measured using LIAISON tTG ($F = 3.39$, $P < 0.05$) or QuantaLite ($F = 7.28$, $P < 0.01$), but not Celikey tTG ($F = 2.69$, $P =$ not significant [NS]) assays.

In the 26 children with a GFD after the diagnosis of CD, anti-tTG levels significantly decreased (Student t test for paired data, $t = 4.99$, $P < 0.001$ for LIAISON; $P = 4.58$, $P < 0.001$ for Celikey tTG and $t = 6.71$, $P < 0.001$ for QuantaLite). The percentage of reduction in anti-tTG levels after a GFD was correlated with the duration of diet only when the QuantaLite levels were considered (Spearman rho = 0.499, $P < 0.05$), but not when LIAISON tTG (Spearman rho = 0.249, $P =$ NS) or Celikey tTG levels (Spearman rho = 0.141, $P =$ NS) were considered.

DISCUSSION

Antiendomysial antibodies and anti-tTGs are the most sensitive and specific serological indices of CD (1–13,16,17,20). Recent studies report sensitivities and specificities of second-generation anti-tTG ELISAs, which use human tissue transglutaminase as antigen, of above 95%, a value that in most cases is higher than values obtained with EMAs (17,29–33). Automated chemiluminescence-based assays for the determination of different analytes, available in current laboratory practice, provide accurate results (23–25). In the present study, we verified that chemiluminescent assay performed on the LIAISON analyser to detect anti-tTGs enhances the diagnostic performance of this test with respect to second-generation ELISAs.

In CD children, anti-tTG mean levels were significantly higher than in controls or in CD children on a GFD. The 2 investigated ELISAs and the chemiluminescent assay showed very high sensitivity and specificity in diagnosis CD. However, the best discriminant assay was the chemiluminescent LIAISON tTG, which showed a sensitivity value of 97% and a specificity value of 98%, also better than EMAs, although both tests detect anti-

TABLE 1. *ROC curve analyses obtained with the 3 anti-tTG assays for comparison between CS and CD children*

Assay	Under ROC curve area (95% CI)	Cutoff	Sensitivity %	Specificity %	DPR %
LIAISON tTG	0.991 (0.979–1.002)	16.9 AU/mL	97.03	98.06	95.1
Celikey tTG	0.977 (0.957–0.996)	4.4 U/mL	94.06	97.06	91.1
QuantaLite	0.988 (0.974–1.002)	48.0 U	98.02	96.12	94.1

The cutoff values reported are those associated with the highest sensitivity and specificity, which were obtained by calculating the differential positive rate (DPR) for each cutoff value from the formula DPR, sensitivity – (1 – specificity). DPR, sensitivity correct to DPR = sensitivity.

TABLE 2. *False-negative and false-positive results obtained with the different studied assays*

False-negative results among CD children					
CD patient no.	EMA	LIAISON tTG (cutoff = 16.9 AU/mL)	Celikey tTG (cutoff = 4.4 U/mL)	QuantaLite (cutoff = 48 U)	Total IgA, g/L
1	−	−	+	−	0.31
2	−	−	−	−	1.26
3	+	−	−	+	2.37
4	+	+	−	+	0.92
5	+	+	−	+	3.42
6	+	+	−	+	0.84
7	+	+	−	+	0.88
8	+	+	−	+	2.37
False-positive results among CS					
Control patient no.	EMA	LIAISON tTG (cutoff = 16.9 AU/mL)	Celikey tTG (cutoff = 4.4 U/mL)	QuantaLite (cutoff = 48 U)	Total IgA, g/L
9	+	−	+	−	0.67
10	+	+	+	+	0.91
11	+	−	−	+	1.03
12	+	+	+	+	1.41
13	+	−	+	+	1.64

Total IgA levels recorded in the single children are also shown.

bodies elicited against the same antigen, tissue transglutaminase (4). In agreement with this observation was the finding of an association between anti-tTG levels and EMA semiquantification. Our data, moreover, confirm the observation that, when measured using the best available methods, the diagnostic performance of anti-tTGs is as reliable as EMAs in diagnosing CD in children (33), as already reported in adults (17,30). On these bases, we suggest that a positive anti-tTG result does not need further confirmation by EMA testing, as proposed by other authors (31,32,34,35). This approach could allow us to overcome the main drawbacks of EMA determination, a technique which cannot be automated, is time consuming and, in part, operator dependent. The excellent performances of the chemiluminescent assay evaluated in this study are probably consequent to the analytical characteristic of the method: the acridinium esters, used in flash chemiluminescence are smaller than an enzyme, used in ELISAs, this favouring the reaction kinetics.

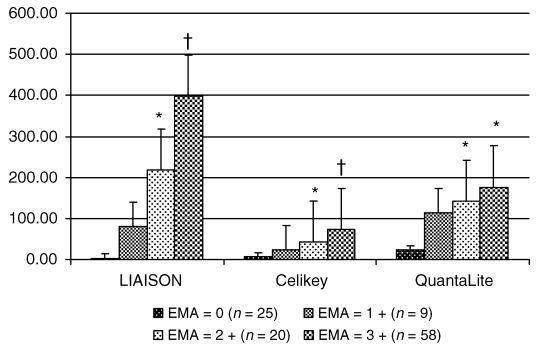

FIG. 2. Mean values (columns) and SDs (bars) of anti-tTGs measured with the 3 different assays tested in this study in children with CD at diagnosis or after GFD after they have been subdivided according to EMAs semiquantification. The number of children belonging to each group is reported in brackets. Bonferroni test for pairwise comparisons: $^{*}P < 0.05$ with respect to EMA 0; $^{\dagger}P < 0.05$ with respect to EMA 0, EMA 1+ and EMA 2+.

Some CD children had EMAs and/or anti-tTG false-negative results. Because both EMAs and anti-tTG are IgA class antibodies, we analyzed whether false-negative results were consequent to total IgA deficiency. Among the 8 false-negative CD children only one had a partial IgA deficiency (0.31 g/L), and this suggests that different pathophysiological conditions other than total IgA deficiency might concur in causing false-negative IgA autoantibodies results in CD. Consequently, as pointed out by us in a previous paper (13), the problem of false negatives cannot be completely solved by IgG assays, and histology must be performed when CD is suspected despite negative serology. Five children without a histologically confirmed CD have false-positive EMAs and/or anti-tTGs. These patients might have potential CD, being at risk for developing typical CD enteropathy later in life (36). However, only a careful follow-up evaluation of these subjects will allow us to conclude about the reliability of the different assays in identifying patients potentially developing CD in their further life.

Celiac disease covers a wide spectrum not only of clinical manifestation but also of intestinal pathological lesions. The Marsh classification has been adopted to describe the progression of the abnormalities in the celiac mucosa (28). Because autoantibodies against tissue transglutaminase and endomysium are produced in the intestinal mucosa in early as well as in advanced CD, we

verified whether the circulating levels of anti-tTGs were correlated with the degree of mucosal lesions. Both EMA semiquantification and anti-tTG levels were correlated with Marsh classification, indicating that as higher are the serological results as worse is probably the degree of intestinal atrophy. In agreement were the findings made on the group of CD children on GFD; anti-tTG, measured with any of the 3 methods investigated in this study, was significantly reduced after a GFD. However, only with the QuantaLite method was the decline in serum levels correlated with the duration of gluten withdrawal, suggesting that anti-tTG quantification made with this test probably mirrors more closely than other methods gluten-induced autoimmunity in CD.

In view of the high performance of anti-tTGs in the diagnosis of CD, a positive laboratory result must refer the patient to the gastroenterologist. Therefore, in agreement with Sinclair and Duncan (37), we suggest that in their anti-tTG–positive reports laboratories should incorporate a recommendation for general practitioners, providing them guidelines on the course of action to take in improving the management of CD.

In conclusion, the determination of anti-tTGs with second-generation ELISAs is as reliable as EMAs determination in the diagnosis of CD. The novel chemiluminescent method described in the present paper for the detection of anti-tTGs in the diagnosis of CD had a higher sensitivity (97%) and specificity (98%) than other methods. The anti-tTG test is suitable for verifying patient compliance to dietary treatment.

Acknowledgments: The authors would like to thank Mr. Flaviano Favaro, Mr. Federico Fior and Mrs. Monica Razetti (Department of Laboratory Medicine, University-Hospital of Padova, Italy) for their technical assistance.

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