

Human Recombinant Tissue Transglutaminase ELISA: An Innovative Diagnostic Assay for Celiac Disease

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OBJECTIVE: Tissue transglutaminase is the autoantigen recognized by the sera of celiac patients. An enzyme-linked immunosorbent assay (ELISA) based on guinea-pig tissue transglutaminase was recently used to measure serum tissue transglutaminase antibodies for the diagnosis of celiac disease. We determine the sensitivity and specificity of an ELISA test based on the use of human recombinant transglutaminase, compared with the guinea pig transglutaminase ELISA and IgA antiendomysium antibodies.

METHODS: Serum samples were tested from 65 patients with intestinal biopsy proven celiac disease, from 10 patients with Crohn's disease, and from 150 healthy blood donors.

RESULTS: Human transglutaminase ELISA identified 64 of 65 celiac patients, whereas the guinea pig transglutaminase ELISA and IgA antiendomysium antibodies identified 58 of 65 and 60 of 65 subjects, respectively. The three tests showed comparable specificity.

CONCLUSIONS: These results proved that the human tissue transglutaminase-based ELISA represents a cost-effective strategy for identifying both symptomatic and atypical forms of celiac disease and could mean that intestinal biopsy need no longer be the gold standard for diagnosing this clinical condition. Furthermore, early identification and treatment of patients with celiac disease in an outpatient setting could have significant implications for reducing long-term morbidity and can produce major savings in future health care costs. (Am J Gastroenterol 2000;95:1253–1257. © 2000 by Am. Coll. of Gastroenterology)

INTRODUCTION

Celiac disease (CD) is an autoimmune enteropathy triggered by the ingestion of gluten in susceptible individuals. The disease is associated with HLA alleles DQ α 1*0501/DQ β 1*0201, and is self-perpetuating with continued exposure to gluten (1). Although initially thought to be relatively uncommon, it has now been found to be one of the most frequent genetically based diseases (prevalence, 1:250) in the general population (2, 3), often manifesting itself by vague nongastrointestinal symptoms (4). During the active phase,

a characteristic IgA autoantibody recognizing the endomysium is produced. These autoantibodies can be identified by an immunofluorescence assay, using either monkey esophagus or human umbilical cord as tissue substrate, with a diagnostic sensitivity of 68–100% and a specificity of 100% when compared with intestinal biopsy (5). The antigen recognized by IgA endomysium antibody (AEA) has recently been identified as tissue transglutaminase (tTG) (6). It has recently been reported that the guinea-pig transglutaminase-based (gp-tTG) enzyme-linked immunosorbent assay (ELISA) used for diagnosis of CD has a sensitivity ranging from 86.6% to 98% and a specificity ranging from 90% to 98% (7–10). However, this test presents limitations that would prevent its use as an alternative to the AEA assay (11). In a recent communication, the IgA gp-tTG ELISA gave false-positive results in as many as 50% of patients at risk for CD with autoimmune hepatitis or primary biliary cirrhosis, due to the presence of hepatic proteins in the commercial tTG obtained from guinea-pig liver (12). One recent report (13) described better results using human tTG. However, the proposed radioisotope-based binding assay, involving *in vitro* translation and immunoprecipitation, is not suitable for routine diagnostic use. Therefore, despite these new diagnostic developments, the intestinal biopsy remains the gold standard assay for the diagnosis of CD. Here we describe the use of human recombinant tTG (h-tTG) in an ELISA that showed a higher sensitivity, compared with both gp-tTG and AEA assays.

MATERIALS AND METHODS

Subjects

Serum samples from 65 untreated, biopsy confirmed CD patients (31 men, 34 women; median age, 12 yr; range, 2–60 yr) diagnosed during the period June 1997 to May 1999, following the revised ESPGHAN criteria for CD (14), were used as the disease group. Forty-seven untreated celiacs were diagnosed on the basis of subtotal villous atrophy and 18 on the basis of total villous atrophy in intestinal specimen. The patients were diagnosed and followed up at the Department of Pediatrics of the "Burlo Garofolo" Chil-

Table 1. Comparison of Sensitivity and Specificity of AEA, gp-tTG, and h-tTG Assays

Groups	n	AEA +	gp-tTG+			h-tTG+		
			IgG	IgA	IgG/IgA	IgG	IgA	IgG/IgA
Patients with CD	65*	60	40	53	58	44	59	64
Sensitivity		93%	61.5%	84%	90%	67.6%	91.5%	98.5%
Patients with Crohn's disease	20	0	0	0	0	0	0	0
Blood donors	150	0	3	3	6	7	1	8
Specificity		100%	98%	98%	96%	96%	99%	95%

* Two subjects with serum IgA deficiency.

AEA = IgA endomysium antibody; gp-tTG = guinea-pig transglutaminase; h-tTG = human recombinant transglutaminase; CD = celiac disease.

dren's Hospital in Trieste (35 CD patients: 17 men and 18 women) and at the Division of Pediatrics, University of Maryland at Baltimore (30 CD patients: 14 men and 16 women). Serum samples from 20 Italian patients with Crohn's disease with normal jejunal biopsy (15 males; five females; age 12–18 yr) and from 150 blood donors (100 Americans and 50 Italians, 80 men, 70 women; age 18–60 yr) were also analyzed for antibodies to gp-tTG, h-tTG, and AEA.

Human Recombinant Transglutaminase

The h-tTG gene was amplified from the intestinal biopsy of a patient with active CD using primers specific for the coding region of the gene (15). The cDNA was cloned into an expression vector (pET28b, Novagen, Madison, WI), expressed in bacteria, and purified under nondenaturing conditions using IMAC (Qiagen, Valencia, CA). Purity of the recombinant protein was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

ELISA

Microtiter plates (EIA/RIA 2580, Costar, Cambridge, MA) were coated with 1 µg h-tTG or gp-tTG (Sigma T5398, lot 18H7820, St. Louis, MO) in 100 µL of 50 mmol/L Tris-buffered saline (TBS) with 5 mmol/L CaCl₂, pH 7.5. The plates were washed 3 times with TBS, 10 mmol/L EDTA, 0.1% Tween 20 (TTBS). Serum samples diluted 1:100 in TTBS were incubated for 1 h at room temperature (RT). The plates were washed, and incubated for 1 h at RT with either 1:3000 phosphatase-conjugated antihuman IgA (Sigma A-3062) or 1:7000 antihuman IgG (Sigma A-8542) diluted in TTBS. The immune reaction was developed by adding substrate solution and the absorbance was read in a microplate reader at 405 nm. The positive control, made by a pool of CD patient's sera positive for tTG, reaches an optical density value of 2 for IgA and 1.5 for IgG in about 30 min. At that point each sample's optical density (OD) is used to calculate the percentage results, based on this formula: [(OD sample – OD blank)/(OD positive control – OD blank)] × 100. Normal values were taken as <13% IgA and <30% IgG for h-tTG, and <7% IgA and <16% IgG for gp-tTG. This value is >2 SD above the mean of 100 healthy Italian subjects tested previously to obtain normal cut-off values (60 males, 40 females, aged 1–16 yr) for both h-tTG and gp-tTG.

Antendomysium Antibody Assay

Serum IgA AEA levels were measured by means of indirect immunofluorescence, using cryostat sections of human umbilical cord, as previously described (16). Briefly, the sections were incubated with the subject's serum diluted 1:5 for 30 min. After washing, sections were incubated with fluorescein-labeled antihuman IgA antibodies for 30 min. The slides were washed and examined by fluorescent microscopy.

The immunological tests were performed by three operators (I.B., C.T., and A.T.) unaware of the clinical and laboratory findings of the subjects tested.

RESULTS

The results of the ELISA on patients with CD, patients with Crohn's disease, and healthy controls are shown in Table 1; h-tTG-based ELISA was positive in all but one of the biopsy confirmed CD patients. When subdivided according to immunoglobulin class, 59 of 65 CD patients were positive by IgA (sensitivity, 91.5%) and 44 of 65 by IgG ELISA (sensitivity, 75.6%). Although the sensitivity of the IgG ELISA alone was rather low, it proved very effective in combination with the IgA ELISA, as it identified five of the six patients negative to the IgA assay, giving a combined sensitivity for the h-tTG ELISA of 98.5% (Table 1). The diagnostic efficiency of the gp-tTG ELISA, on the other hand, was lower, with a sensitivity of 84.4% for IgA (53/65), 72.2% for IgG (40/65), and 90.2% for IgA and IgG combined (58/65). Even though the AEA assay identified two CD patients who were missed with the gp-tTG ELISA (60/65), its sensitivity (93%; 60/65) was still lower than that of the h-tTG ELISA (98.5%; 64/65).

Of the CD patients testing positive by h-tTG ELISA and negative by either AEA or gp-tTG ELISA, 11 of 12 complained of the symptoms typical of CD. Two of the five celiacs who tested negative by AEA had serum IgA deficiency with total villous atrophy, whereas the other three had normal serum IgA levels and subtotal villous atrophy (Table 2).

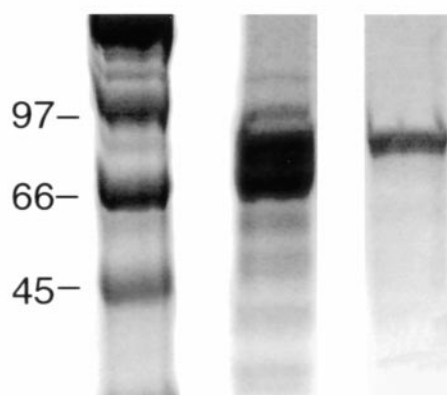
All 20 patients with Crohn's disease were negative for both the gp-tTG and h-tTG ELISA tests (Table 1). Of the 150 healthy controls, seven were identified as positive by h-tTG IgG ELISA (specificity, 95.5%) and one by IgA

Table 2. Clinical Features and Degree of Histological Abnormality in Intestinal Biopsies of Untreated Celiac Patients Identified Using h-tTG ELISA, But Not Using Either Guinea Pig-tTG ELISA or IgA Antiendomysium Antibodies

Age (Yr)	Gender	Clinical Findings	gp-tTG ELISA	IgA AEA	HLA Type	Intestinal Biopsy
1	M	Failure to thrive	+	—	DQ α 1, β 1	Subtotal VA
9	F	Down's syndrome, recurrent abdominal pain, total serum IgA deficiency	+	—	ND	Total VA
24	F	Total serum IgA deficiency, daughter of type-1 diabetic subject	+	—	DQ α 1, β 1	Total VA
45	F	Anemia, dental enamel defects	+	—	DQ α 1, β 1	Subtotal VA
64	F	Anemia, Sjogren's syndrome	+	—	DQ α 1, β 1	Subtotal VA
2	F	Recurrent abdominal pain	—	+	DQ α 1, β 1	Subtotal VA
6	F	Diarrhea, failure to thrive	—	+	DQ α 1, β 1	Subtotal VA
7	F	Recurrent abdominal pain	—	+	ND	Subtotal VA
8	M	Diarrhea	—	+	ND	Total VA
9	M	Diarrhea	—	+	ND	Subtotal VA
30	M	Anemia	—	+	ND	Subtotal VA
35	F	Asymptomatic, mother of children with CD	—	+	ND	Subtotal VA

ELISA = enzyme-linked immunosorbent assay; VA = villous atrophy; M = male; F = female; CD = celiac disease; ND = not done; other abbreviations as in Table 1.

ELISA (specificity, 99%), whereas with gp-tTG ELISA the specificity was 98% with both IgA and IgG (Table 1). The six gp-tTG-positive healthy subjects were also h-tTG positive. At this stage, we cannot establish whether these six subjects were truly healthy or asymptomatic celiacs, because the samples from blood donors were covered by confidentiality that prevented us from proposing an intestinal biopsy to confirm the diagnosis. All healthy controls and all patients with Crohn's disease were found to be negative by AEA (Table 1). The better performance of the h-tTG ELISA, compared with the gp-tTG assay, may be related to the purity of the recombinant human transglutaminase, compared with commercially available guinea-pig protein. As shown in Figure 1, the h-tTG recombinant protein appeared as a single band of 82 kD on SDS-PAGE, whereas the gp-tTG showed multiple bands, probably due to contamination and/or protein degradation.

**Figure 1.** SDS-PAGE analysis of both guinea-pig transglutaminase protein (left lane) and recombinant human transglutaminase protein (right lane). Positions of molecular weight standards are indicated.

DISCUSSION

In this paper we described the development of an innovative human-based tTG ELISA, which allowed us to identify a total of 12 CD patients missed with the other two currently available noninvasive serological tests (seven patients missed by gp-tTG and five missed by AEA). These results support previously reported concerns regarding the use of nonhuman tTG as an alternative test to the AEA assay (10, 11), and may explain the small but consistent number of patients with CD who test negative by AEA. In our study, two of these patients were affected by IgA deficiency, a condition often associated with CD (17), and the remaining three had low specific anti-h-TG IgA antibodies (13%, 14%, and 20%). The less sensitive subjective AEA assay may have missed the latter three cases precisely because of the low quantity of antibody in the serum. Of note, all five of these patients showed high levels of specific anti-h-tTG IgG (40%, 45%, 56%, 75%, and 97%).

Our data emphasize the diagnostic value of h-tTG IgG antibodies, which were able to identify all the celiac subjects missed by both IgA AEA and IgA h-tTG. These antibodies not only increase the sensitivity of the test to levels comparable to the gold standard, intestinal biopsy (see earlier), but also make it possible to identify CD patients with IgA deficiency whose immunological defect (often complicated by recurrent infections) may be corrected by a gluten-free diet (17).

The partial purity of the gp-tTG (see Fig. 1) may account for the variability in sensitivity and specificity of the gp-tTG ELISA reported in the literature (7–10). Furthermore, the better results obtained with h-tTG may also reflect the 7% amino acid difference between h-tTG and GP-tTG, suggesting that more than one epitope is recognized by patients' antibodies. This hypothesis is supported by our preliminary results obtained with anti-h-tTG single chain antibodies selected from a phage library derived from CD patients, show-

ing that some clones recognize both gp-tTG and h-tTG, whereas others recognize only the human form (18).

The h-tTG-based ELISA offers a series of advantages over the currently available diagnostic tests for CD. The routine use of the AEA assay is limited by elevated costs, a time-consuming protocol unsuitable for testing large numbers of samples, and use of the esophagus of an endangered species (such as the monkey) as the substrate for the immunofluorescence analysis. Even if we (3) and others (19, 20) resolved this last issue by using human umbilical cord as a valid alternative to monkey esophagus, it has been recently reported that the subjective interpretation of the AEA assay may lead to unacceptable variability among laboratories that perform this test (21). The h-tTG ELISA also offers indisputable advantages over the costly, time-consuming, and invasive intestinal biopsy. Thanks to its high sensitivity and specificity, and its low costs, compared with presently used diagnostic methods, this simple test may be considered an excellent diagnostic tool for both symptomatic and asymptomatic CD patients. Furthermore, the higher sensitivity of our IgA-IgG h-tTG ELISA, compared to IgA AEA, might address the recently reported limitations of the IgA-AEA test in untreated celiac patients with partial villous atrophy (Marsh IIIa criteria), infiltrative lesion (Marsh I criteria), or hyperplastic lesion (Marsh II criteria) of the jejunal intestine (22). In these conditions, it may be that the related celiac antibodies are much more present in jejunal fluid than in the serum specimens (23), and that, therefore, the low quantity of these antibodies in the serum failed to be identified by low-sensitivity immunological techniques such as indirect immunofluorescence assay.

If our results are confirmed on a large scale, it is possible to predict that the diagnosis of CD will be reduced to a simple ELISA test for almost all patients, with significant advantages in terms of savings for the community, less discomfort for the patients, and reduced risk of the serious complications, such as intestinal lymphoma (24) or other autoimmune diseases (25), arising from prolonged gluten exposure.

ACKNOWLEDGMENT

This work was supported by grant 46/98 from Istituto per l'Infanzia I.R.C.C.S. "Burlo Garofolo," Trieste, Italy.

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Received Aug. 17, 1999; accepted Jan. 6, 2000.

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