Diagnostic value of various serum antibodies detected by diverse methods in childhood celiac disease

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The diagnostic performances of antiendomysium IgA detected on monkey esophagus and human umbilical cord smooth muscle, of antireticulin IgA, and of antigliadin IgA and IgG were calculated in 74 children with celiac disease (CD) or other gastrointestinal disorders. We also compared four methods for gliadin antibody detection. With a diagnostic specificity of 100%, diagnostic sensitivity was 94% for antireticulin IgA, 93% for antiendomysium IgA when detected on human umbilical cord smooth muscle, and 97% when detected on monkey esophagus. The diagnostic sensitivity for gliadin antibody was highest with an ELISA procedure, followed by fluorogenic detection (94% for IgG, 91% for IgA, 97% with IgA and IgG combined). Because of its high diagnostic sensitivity and ease and speed of use, the combined antigliadin IgG and IgA antibody assay is suitable for screening large groups of patients. In IgG- or IgA-positive cases, the more demanding and more specific antiendomysium IgA evaluation is required to confirm suspected CD.

INDEXING TERMS: umbilical cord • endomysium • gliadin • reticulin

Celiac disease (CD) is diagnosed from such typical histological lesions as total or subtotal intestinal villous atrophy and crypt hyperplasia evaluated on jejunal biopsies [1,2]. However, when clinical signs suggest CD, the first diagnostic approach is the evaluation in serum of the antigliadin (AGA), antiendomysium (EMA), and antireticulin (ARA) antibodies, high titers being indicative of active CD [3,4]. Thus, there is a need for simple, efficient methods with which to evaluate these antibodies, not only to diagnose patients presenting with symptoms of CD, but also to monitor the efficacy of a gluten-free diet, and for population screening to detect the clinically silent form of the disease [5–7]. The diagnostic efficiency of the methods used at present to evaluate antibody titers differs according to the prevalence values of the disease, the patient’s age, and the methodologies used [3,8,9].

We evaluated, in a case-control retrospective study, a variety of immunological methods, by analyzing AGA, ARA, and EMA concentrations in sera from 32 untreated CD patients and from 42 patients affected by other gastrointestinal disorders. The aim of this study was to establish in our population of children from Campania (southern Italy): (a) which AGA test was the easiest to perform, the least time consuming, and the most efficient; (b) if the use of human umbilical cord smooth muscle, instead of monkey esophagus, as antigen-containing tissue for EMA detection enhanced the diagnostic power of the test in childhood CD, as recently reported for adult CD [10]; and (c) the most efficient sequential immunological approach for CD diagnosis.

Patients and Methods

We examined sera from 74 children from the Department of Pediatrics of our University School of Medicine. The Department houses a center for the study of CD and other gastrointestinal disorders, which explains the high prevalence of CD in the studied sample. The patients were divided into two groups: (a) 32 children, median age 5 years, in whom the diagnosis of active CD was established according to the criteria of the European Society of Paediatric Gastroenterology and Nutrition [1,2], all having subtotal or total villous atrophy at the first intestinal biopsy; (b) 42 children, median age 2 years, with other gastrointestinal diseases, but whose symptoms (growth failure, weight loss, diarrhea, vomiting, irritable bowel, abdom-
inal distension, sideropenic anemia) were compatible with a diagnosis of CD. All patients whose clinical symptoms and laboratory tests (xylose test, sugar permeability test) were suggestive of CD underwent intestinal biopsy. No patient in either group had selective IgA deficiency.

SAMPLES
All samples were centrifuged immediately after collection, and within a few hours sera were examined for the presence of IgG AGA, IgA AGA, and IgA EMA. They were then subdivided into aliquots and stored frozen at −80 °C until required for the other tests. No test was performed on specimens that had been frozen and thawed more than once.

EMA ASSAY
We used two indirect immunofluorescence methods to detect IgA EMA: (a) the classical EMA assay (Anti-Endomisio®; Eurospital, Trieste, Italy), in which serum is diluted 1:5 with phosphate buffer and sections from the distal portion of monkey esophagus are the antigen-containing substrates; and (b) an assay in which custom-made cryostatic sections of human umbilical cord are the antigen-containing tissues [10].

ARA ASSAY
IgA ARA were determined with an indirect immunofluorescence method on tissue sections of rat kidney, with sera diluted 1:2.5 with phosphate buffer (ARA kit; Immco Diagnostics, Buffalo, NY).

AGA ASSAYS
We used four procedures to evaluate AGA. Method 1 (Gluten IgA and IgG EIA; Kabi Pharmacia Diagnostics, Uppsala, Sweden) and method 2 (α-Gliastick® IgA and IgG, Eurospital) are ELISAs for the measurement of IgA and IgG antibodies. With method 1, total gliadin is the antigen, 1:201 sera dilutions are used, and the immunocomplex is detected on a chromogenic substrate. The manufacturer's upper reference limits (obtained from the examination of 895 patients affected by celiac and other gastrointestinal disorders) for IgA and IgG are 35 arbitrary units (AU) for patients ages 0–4 years and 20 AU for patients >4 years old. With method 2, purified α-gliadin is the antigen, sera are diluted 1:201, and the immunocomplex is detected by a fluorescence measurement; the manufacturer's upper reference limits (obtained from the examination of ~1200 subjects: controls, celiac, and other gastrointestinal patients) are 7 AU for IgA and 15 AU for IgG assays. Methods 3 and 4 measure both total AGA (Ig): Method 3 (Gliastick®, Eurospital) is an ELISA method, described by Cavataio et al. [11], in which, in presence of AGA, color develops on sticks bearing adsorbed wheat gliadin in reactive pads; method 4 (AGA; Biosystems, Barcelona, Spain) is an indirect immunofluorescence procedure in which rat kidney tissue is incubated with gliadin before addition of sera diluted 1:40.

QUALITY-CONTROL PROCEDURE
The two ELISA methods (methods 1 and 2) used for AGA evaluation include the examination, together with patients' samples, of calibrator and control samples: one below the upper reference limit and one at 100 AU. Both methods had a linearity range up to 100 AU and the average CV was <10%. The sticks used for AGA evaluation (method 3) carried two negative control pads alternated with the reactive pads.

All immunofluorescence assays [AGA, ARA, antiubilical cord antibody (UCA), and EMA] include assays of negative and positive control sera simultaneously with the assays of patients' samples. All immunofluorescence readings were performed independently by two observers, and no equivocal result was obtained.

STATISTICAL ANALYSIS
Spearman's rank test [12] was used to evaluate the correlation between AGA concentrations and patient age. Diagnostic characteristics (sensitivity, specificity, and efficiency) were used and calculated according to Galen and Gambino [13], at the cutoff values indicated in the diagnostic kits. McNemar's test [14] was used to compare measurements of the same variable obtained with different procedures, e.g., IgA and IgG with method 1 vs IgA and IgG with method 2; total Ig with method 3 vs total Ig with method 4; EMA vs UCA and ARA; UCA vs ARA. This statistical test quantifies the probability that the differences of the results obtained with two different methods are due to chance rather than to a real difference between the procedures.

Receiver-operating characteristic (ROC) plots [15] were used to compare the diagnostic features of IgA AGA and IgG AGA tested by methods 1 and 2, at different cutoff points; ROC plots also showed which antibodies, IgA or IgG, had the highest discriminating power for CD vs non-CD gastrointestinal disorders. Statistical analysis was performed on an IBM PC, using the SPSS statistical package [16], and the CLABROC program [17].

INSTRUMENTATION
The EASIA System (Medigenix Diagnostics, Brussels, Belgium) and the FSA fluorometer (Eurospital) were used for ELISA measurements in methods 1 and 2, respectively. A fluorescence microscope (Zeiss, Oberkochen, Germany) was used for the fluorescence detection of AGA (method 4), ARA, EMA, and UCA.

Results
No false-positive results were obtained with any of the three procedures used to assay EMA, UCA, and ARA (100% diagnostic specificity) in the control group of 42 children affected by gastrointestinal disorders other than CD (Table 1). In the 32 celiac-affected children, there was one false-negative result with EMA (this patient was negative also with UCA and ARA), whereas two false-negative results were obtained with UCA and with ARA in the same two patients (Table 1). Therefore, the degree of concordance was 98% between EMA and UCA and between EMA and ARA, whereas it was 100% between UCA and ARA tests. Obviously, because the three methods are based on indirect immunofluorescence, the results obtained were on a "yes" or "no" basis.

Spearman's rank test, used to test the correlation between AGA results obtained with method 1 and method 2 and patient
age, showed a slight trend to higher AGA values in the younger subjects, but there was no statistically significant correlation in either control or celiac patients. Consequently, AGA concentrations do not appear to be age dependent.

The diagnostic efficiency of the four procedures used to assay AGA ranged from 80% to 89% (Table 2). IgG AGA had the highest diagnostic sensitivity and IgA AGA the highest diagnostic specificity, each when evaluated with method 2 (see Patients and Methods). McNemar’s test showed concordance between the results obtained with the four AGA procedures; the concordance was lowest (84%) with the procedures that measure total Ig AGA, and highest with the ELISA methods for both IgA AGA (95%) and IgG AGA (92%). With the combined IgA AGA and IgG AGA measurements, diagnostic sensitivity increased to 91% (method 1) and 97% (method 2).

Of methods 1 and 2, method 2 had better global diagnostic efficiency in terms of diagnostic sensitivity for IgG and diagnostic specificity for IgA at the cutoff indicated by the manufacturer. However, we examined the data relative to AGA evaluation with methods 1 and 2 after ROC plot analysis to determine whether the use of a cutoff concentration different from that indicated by the manufacturer could enhance the diagnostic sensitivity of these immunological tests (Fig. 1). We found that: (a) a cutoff of 2.5 AU for IgA estimation with method 2 gave a much better diagnostic sensitivity than the manufacturer’s cutoff of 7.0 AU (91% vs 78%), while the specificity decreased only very slightly; (b) taken together, IgG and IgA assays correctly classified more patients with method 2 than with method 1; and (c) for IgG AGA evaluation, the manufacturer’s cutoff value gave the highest diagnostic sensitivity. Only one case of CD eluded detection when both IgG and IgA were evaluated with method 2 (Fig. 2). This single false-negative case in our population sample was a 23-month-old child who was also falsely negative with all the other immunological tests used; jejunal biopsy confirmed CD in this patient.

**Discussion**

The incidence of CD in Europe varies considerably, mainly because many cases elude detection. In CD patients in southern Italy, the gastrointestinal symptoms are frequently observed at first clinical examination and thus the physician is alerted to the possibility of CD.

The working group of the European Society of Paediatric Gastroenterology and Nutrition has included serum immunological markers among the diagnostic criteria for CD in children [2], one of the aims being to limit the number of intestinal biopsies performed on patients. High diagnostic specificities have been reported for both ARA and EMA, the latter marker being more sensitive [4, 18–20], and EMA detection has even been advocated as a noninvasive alternative to jejunal biopsy, particularly in children, and as a general screening procedure [9]. However, the high cost of the EMA test, the need for highly specialized operators, and the time required to perform the test itself limit its use in screening, as does the presence of some
false-negative results [4, 18, 20]. Recently, the potentially more easily available and less expensive human umbilical cord tissue has been proposed as an alternative antigen substrate to monkey esophagus smooth muscle for EMA detection in adult and childhood CD [10, 21].

The diagnostic specificity we obtained for CD of UCA, EMA, and ARA agrees with earlier reports [4, 10], but we obtained a slightly lower diagnostic sensitivity with UCA than in the adult and pediatric population reported so far [10, 21]. Taken together, these data endorse the continuation of multicenter evaluations of the use of human umbilical cord as tissue antigen for EMA detection as a substitute for monkey esophagus. Our ARA data are consistent with previous results [4, 18, 19] and the diagnostic performance of the test is slightly lower than that of the EMA assay. The concordance between the three methods (evaluated one vs the other) demonstrates that the UCA and ARA assays are totally superimposable. However, when UCA vs EMA and ARA vs EMA were compared, we obtained one case of discordance, which was the same case in both comparisons. These data, even given the slightly better diagnostic sensitivity of EMA, support the reliability of UCA, EMA, and ARA tested by immunofluorescence procedures for the diagnosis of CD.

Various techniques are used to measure AGA in serum, and their diagnostic characteristics differ depending on the class of antibody to detect, the cutoff value used to discriminate controls from celiac patients, the antigen used, and the population examined [4, 8, 9, 11, 19, 20]. Among the four procedures evaluated in this paper, the stick micromethod (method 3) showed a diagnostic efficiency of 87%, with a diagnostic sensitivity lower than that reported by others (90% vs 97%) [11]. This technique is easy to perform and does not require instrumentation; however, it is not fast and thus is not very suitable for screening a large number of patients. The indirect immunofluorescence assay of AGA (method 4) had a diagnostic efficiency of 89%, with a diagnostic sensitivity of 87%, but the technique requires highly experienced personnel, and it is obviously not a rapid

![ROC plots obtained for gliadin antibodies: (A) IgA, (B) IgG.](image)

Method 1, O; method 2, □. The dashed arrows indicate the manufacturer’s cutoff (c.o.); the solid arrow the cutoff selected to improve the diagnostic sensitivity of the test. Two cutoffs are given for method 1 depending on patient age (see Patients and Methods). Areas under the ROC plots and confidence intervals are: for IgA (A) 0.95 ± 0.05 and 0.96 ± 0.04 (two-sided P value not significant), and for IgG (B) 0.89 ± 0.07 and 0.93 ± 0.06 (two-sided P value = 0.01) for method 1 and method 2, respectively.

![Diagnostic sensitivity of the evaluation of IgG+IgA AGA, at different cutoff (c.o.) values, in CD.](image)
method; thus it is more useful as a support than as a routine test. The ELISA procedures (methods 1 and 2) are the most rapid, cheapest, and most easily applicable to large screening programs. In accordance with other reports [3, 4], we found that IgA AGA are more specific, and IgG AGA are more sensitive in detecting CD when the manufacturer’s cutoff concentrations were used for the comparison. However, with a prevalence of the two classes of diseases (celiac and other gastrointestinal diseases used as controls) of ~50% each in the sample population of our cohort at the Childhood Coeliac Unit of our University Hospital, we found that for IgA AGA a cutoff value selected after ROC curve analysis [2.5 AU instead of 7 AU (method 2)] enhanced diagnostic sensitivity from 78% to 91%. A cutoff <2.5 AU decreased the diagnostic specificity (from 95% to 79% when the cutoff was 1.5 AU) without significantly increasing sensitivity; moreover, the cutoff value of 1.5 AU was too near the detection limit of the method. The parallel estimation of IgG and IgA increased the global diagnostic sensitivity of the AGA test to 97% (method 2), and detected also celiac patients affected by IgA deficit.

In conclusion, our data suggest that the evaluation of IgG+IgA AGA by ELISA with fluorogenic detection is the most suitable test for the first diagnostic approach to CD and for screening purposes, because in addition to its diagnostic performance, it is rapid, relatively cheap, and easy to perform. The EMA immunofluorescence assay is better used as a confirmatory test because although it has a diagnostic specificity of 100%, it requires highly skilled personnel and is more expensive and more time consuming than the ELISA procedures.

This sequential approach for the in vitro immunological diagnosis of CD could be useful for screening general populations and groups where the prevalence of the disease is expected to be low. However, the jejunal biopsy remains the gold standard procedure (a) in subjects with positive immunological results in whom confirmation of a diagnosis requiring a lifelong gluten-free diet is necessary, and (b) in subjects with negative immunological results (~3% in our study) when there is a strong clinical suspicion of CD. Finally, the proposed strategy can be considered an alternative to the invasive approach when this procedure has to be avoided for clinical considerations.

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