Detection of Autoantibodies Against Actin Filaments in Celiac Disease

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Introduction: Serum autoantibodies specifically directed toward intracellular cytoskeletal actin filaments (anti-actin antibodies, AAA) were found to be associated with intestinal villous atrophy (IVA) in celiac disease (CD). The aim of this study was to assess IgA-AAA with a commercial test that uses sections of rat intestinal epithelial cells in a well-selected cohort of patients and to evaluate the relationship between the presence of serum IgA-AAA and the severity of intestinal mucosa damage. **Materials and Methods:** Serum samples from 70 CD patients and 150 controls subjects were analyzed retrospectively for the presence of IgA-AAA. **Results:** The indirect immunofluorescence test that we used has a specificity of 100%; the sensitivity of the test is not high (25.7%). In this study we also show that serum AAA are more frequently positive in CD patients with total IVA (77.8%) and that

this association is significant. **Discussion:** IgA-AAA certainly cannot take the place of much more sensitive tests such as a-tTG and EMA in the diagnosis of CD because of their low sensitivity; nonetheless, these antibodies could be determined in a-tTG and/or EMA positive patients who cannot undergo an intestinal biopsy because of a severe contraindication, or in the case of negative consensus regarding endoscopy, or when the histology interpretation is difficult. **Conclusion:** In conclusion, the IFI commercial test with intestinal epithelial cells as substrate offers a useful method for IgA-AAA determination. Serum IgA-AAA positivity is indicative of more severe intestinal histology damage and their assay could be a real help to the clinician, especially in the complicated cases. J. Clin. Lab. Anal. 27:21–26, 2013. © 2012 Wiley Periodicals, Inc.

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INTRODUCTION

Celiac disease (CD) is a syndrome characterized by damage to the small intestinal mucosa caused by the gliadin fraction of wheat gluten and similar alcohol-soluble proteins of barley and rye in genetically susceptible subjects (1). A cascade of gluten-induced immune reactions is associated with the appearance of serum specific antibodies and with intestinal mucosal lesions (2–5); moreover, the involvement of the cytoskeleton (6) has also been reported in CD: gluten challenges have been shown to cause the rapid disappearance and disorganization of actin filaments on the intestinal mucosa of CD patients (7, 8). Filamentous actin (F-actin) is known as the major component of microfilaments (MF) of cell cytoskeleton and it is regarded as the target of a subspecificity of

smooth muscle antibodies (SMA) of immunoglobulin G (IgG) class, closely associated with type-1 autoimmune hepatitis (AIH-1). Clemente et al. (9) described serum autoantibodies of the immunoglobulin A (IgA) class specifically directed toward actin filaments (antiactin antibodies, AAA) in CD patients using indirect immunofluorescence (IFI) and laryngeal carcinoma cells (HEp-2) as a

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TABLE 1. Patients' Histological Type, at the Time of the Diagnosis, According to Marsh–Oberhuber

Histological type	Adults	Children	Total cases
Type 1	1	0	1
Type 3a	4	4	8
Type 3b	16	7	23
Type 3c	20	18	38

substrate. This immune reaction was found to be associated with intestinal villous atrophy (IVA). To improve the sensitivity of the IgA-AAA immunofluorescence test, Clemente et al. (10) used another substrate instead of the HEp-2 cells or fibroblasts used in her previous study (9). IgA-AAA were analyzed by immunofluorescence using a newly developed method based on intestinal epithelial cells cultured in the presence of colchicines. This method showed better performances than the previously described test (9).

The aims of this study were (a) to assess IgA-AAA in a well-selected cohort of patients with a commercial test which uses this method; (b) to evaluate the relationship between the presence of serum IgA-AAA and the severity of intestinal mucosa damage.

MATERIALS AND METHODS

Celiac Patients

Serum samples from 70 CD patients (41 adults, 31 females, 10 males, age range: 16–76 years; 29 children, 18 females, 11 males, age range: 2–14 years) were analyzed retrospectively for the presence of IgA-AAA. Antitransglutaminase (a-tTG), antiendomysial antibodies (EMA) positivity, and abnormal intestinal histology were the required inclusion criteria. Intestinal biopsies were classified according to the Marsh classification modified by Oberhuber et al. (11). Histological diagnosis of CD patients are reported in Table 1. Antigliadin antibodies (AGA) positivity were a further required inclusion criteria for children aged ≤ 5 years (12).

Control Groups

Serum samples from 150 subjects (96 females, 54 males, age range 17–85 years), of whom 126 disease controls and 24 healthy controls were analyzed retrospectively for the presence of IgA-AAA.

Disease controls

Diagnosis for 126 disease controls included viral hepatitis/cirrhosis (no. 31), irritable bowel syndrome (no. 15), gastroesophageal reflux (no. 10), ulcerative colitis (no. 10),

cows' milk and food allergy (no. 18), Crohn's disease (no. 12), unspecific colitis (no. 6), nonulcerative dyspepsia syndrome (no. 9), AIH (no. 15, SMA and IgG-AAA positive). Disease controls were a-tTG and EMA negative. Histological diagnosis of 25 subjects was also known: 15 biopsies were normal, while 10 showed histopathology 1.

Healthy controls

Serum samples were obtained from blood donors who underwent CD serologic screening tests and in whom the diagnosis of CD was excluded on the basis of negative a-tTG and EMA serology.

IgA-AAA Detection by IFI

Serum AAA assays were performed with an IFI test (Eurospital, Trieste, Italy) that uses sections of rat intestinal epithelial cells. Serum samples (stored at -20°C until analysis of IgA-AAA) were diluted 1:5 with sample diluent and incubated at 56°C for 30 min (to inactivate thermolabile F-actin depolymerizing factors) such as positive and negative controls. A total of $40\ \mu\text{L}$ of incubated controls and $40\ \mu\text{L}$ of each diluted sample were dispensed into sections. The slides were placed in a moist chamber and incubated at room temperature for 30 min. After incubation, the slides were incubated for 5 min at room temperature with washing solution, then the washing solution was changed and the incubation was repeated for 5 min. A total of $30\ \mu\text{L}$ of sheep anti-human IgA labeled with isothiocyanate fluorescein (FITC) was then added to each section. The slides were placed in the moist chamber and incubated for 30 min at room temperature. After incubation, the washing procedure was repeated, the liquid drops were removed, and a few drops of mounting medium were added to each section.

Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the IgA-AAA were calculated. Associations between the positivity of IgA-AAA, histological characteristics, and age groups were measured using the chi-square nonparametric test with Yates' correction. A nonparametric test of the median was used to compare the average levels of a-tTG and AGA antibodies in the groups of subjects who were positive or negative for F-actin. Tests were considered significant if $P < 0.05$.

RESULTS

Table 2 reports the sensitivity, specificity, PPV, and NPV of test. Positive for IgA-AAA were 18 of the 70 (25.7%)

TABLE 2. Sensitivity, Specificity, PPV, and NPV of Test

	IgA-AAA
Sensitivity (95% CI)	25.7% (24.69–26.74%)
Specificity (95% CI)	100% (100–100%)
PPV (95% CI)	100% (100–100%)
NPV (95% CI)	74.26% (73.65–74.86%)

CD patients, 12 adults and 6 children. None of the controls was IgA-AAA positive.

Table 3 reports the percentage of IgA-AAA positivity and negativity with respect to histological types in CD patients. Among the IgA-AAA-positive patients, 14 (77.8%) were of histological type 3c (nine adults and five children), and this association was found to be statistically significant ($P < 0.041$). In the subgroup 3a–3b, IgA-AAA were positive only in four (22.2%) patients (three adults and one child), which had subtotal IVA (type 3b) and in none of patients with partial IVA (type 3a). Among the CD patients negative for IgA-AAA, there was a much more even distribution of the different degrees of IVA in comparison to that observed in patients who were positive for IgA-AAA (51.9% with 3a–3b, and 46.1% with 3c).

Evaluating the levels of IgA a-tTG and IgG a-tTG in IgA-AAA-positive CD patients, they did not display particularly different levels from those found in IgA-AAA-negative patients. In both cases, the mean value of the antibodies was not significantly different between the two groups of patients positive/negative for IgA-AAA (Table 4). The results obtained for IgA AGA and IgG AGA antibodies (only for CD patients ≤ 5 years) seem to highlight higher levels of AGA antibodies in the IgA-AAA-positive group of patients, although the difference was not statistically significant. Nonetheless, it should be noted that the small number of patients for whom AGA levels have been determined (only 8 out of 70 patients with CD) certainly did not favor the precision of the statistical test.

TABLE 3. Percentage of IgA-AAA Positivity and Negativity With Respect to Histological Types

	Subgroups	IgA-AAA		Total
		Negative	Positive	
Histological characteristics	Type 1	1 2%	0 0	1 1.42%
	Type 3a–3b	27 51.9%	4 22.2%	31 44.3%
	Type 3c	24 46.1%	14 77.8%	38 54.3%
	Total	52 100.0%	18 100.0%	70 100.0%

TABLE 4. Levels of a-tTG and AGA in CD Patients Positive or Negative for IgA-AAA

	IgA-AAA	
	Negative	Positive
IgA a-tTG	81.52 $\pm$ 25.65	90.42 $\pm$ 23.65
IgG a-tTG	40.15 $\pm$ 30.25	52.26 $\pm$ 32.02
IgA AGA ^a	52.36 $\pm$ 46.99	83.20 $\pm$ 23.76
IgG AGA ^a	22.17 $\pm$ 8.66	35.50 $\pm$ 44.55

^aFor CD children aged ≤ 5 years.

Taking the age of the patients into consideration (Table 5), the percentage of IgA-AAA positivity was higher among adults than children (66.7%, compared to 33.3%), while the percentage of IgA-AAA negativity was more homogeneous (adults: 55.8%, children: 44.2%). However, this association did not prove to be significant.

DISCUSSION

SMA of the IgG class with antiactin specificity (SMA T and SMA G) are commonly regarded as markers of AIH-1 (13, 14). Different techniques have been used for their identification, but IFI on rat tissue sections remains the most common method used as first-level test in diagnosing AIH-1. In cases of SMA positivity, a positive confirmation of AAA with specific methods increases the predictive value for AIH-1 (14). Similar AAA belonging, however, to the IgA class, have also been reported in CD and also in this case different methods have been used for their identification (9, 10, 15, 16). In this study, we used an IFI commercial test on rat epithelial intestinal cells to determine the presence of IgA-AAA in CD patients. Our results shows that this test has a specificity of 100%, with the control group testing negative for IgA-AAA, despite our having selected a “critical” control group, essentially comprised disease controls, in which AIH patients were positive for IgG-AAA, but negative for IgA-AAA. The specificity that we obtained was even higher than that of Clemente et al. (10) who achieved 95.1%. The sensitivity

TABLE 5. IgA-AAA Positivity in Relation to Age Groups in CD Patients

		IgA-AAA		Total
		Negative	Positive	
Age group	Adult	29 55.8%	12 66.7%	41 58.6%
	Children	23 44.2%	6 33.3%	29 41.4%
Total		52 100.0%	18 100.0%	70 100.0%

of the test was not high (25.7%) and was lower than in the work of Clemente et al. (10) who reports a sensitivity of 83.9% for the prospective study and of 42.8% for the retrospective study. In the author's opinion, the much lower percentage of IgA-AAA positivity in the retrospective study was due to the fact that IgA-AAA positivity can be significantly affected by serum storage conditions; for this reason Clemente suggests that fresh serum samples should be used for optimal results. In our retrospective study, we observed a lower IgA-AAA positivity rate: this is probably due to the serum storage conditions used in our laboratory (we frozen samples at -20°C for several months, condition probably different from that used by Clemente), even though we avoided repeated freezing and thawing of sera, adhering to the guidelines of the company. On the contrary, fresh samples of patients underwent CD serologic screening tests and IgA-AAA analysis (data not reported) displayed a higher percentage of IgA-AAA positivity than obtained on frozen samples, confirming that specimens should be assayed after collection; in any case, also on fresh samples we obtained a percentage of IgA-AAA positivity still lower than that of Clemente, probably due to the fact that, also if Clemente used the same, our substrate, rat intestinal epithelial cells, the author used a "home-made" method, and thus the treatment of the cells, the conjugate and assay conditions were different respect to the commercial kit that we used. Also Granito et al. (15) used a "home-made" method for IgA-AAA and, compared to the report by Clemente et al. (10), found a lower prevalence of AAA in CD patients, with variations depending on the substrate used (27% using fibroblasts, 25% with HEp-2), but with values closer to those we obtained. Fabbro et al. (16) showed that the chemical and physical treatment of celiac sera samples, as heating or chelating, improved the sensitivity of a "home-made" method with HEp-2 cells as substrate (from 48% to 82%). Bazzigaluppi et al. (17) tested the same commercial test that we used and obtained a sensitivity of 42.68% on fresh serum samples and a specificity of 100%. All these studies show that IFI tests used for IgA-AAA determination have wide ranges of sensitivity and specificity, probably due to the different substrates and conditions used and to the fact that these assays are highly operator-dependent (18), but even when is used method nonoperator-dependent as immunoenzymatic assay (ELISA), sensitivity and specificity values still range from 80% to 87% and 72% to 100%, respectively (15, 19–21). Carroccio et al. (22) compared the IFI assay with ELISA for IgA-AAA determination and found for IFI a sensitivity of 93% higher than value obtained with ELISA (86%). Specifically, the different performances of the ELISA and IFI methods can be explained not only by different analytical characteristics, but also by the presence of contaminant actin in monomeric form (G-actin)

or the use of G-actin as antigen in ELISA, which could interfere with the quality of the test ELISA (9, 15). Another problem is the selection of subjects: Carroccio et al. (19), who evaluated the clinical usefulness of serum IgA-AAA ELISA in CD patients, showed a sensitivity of 80% and a specificity of 85%, which decreased to 72% when only the disease controls were considered as the control group, confirming that the different results obtained by various studies may be also due to the different criteria applied to the selection of subjects, as well as the different performance of the methods used.

In this study we also show a prevalence of AAA in adult CD patients, as reported by other authors (9, 23). Furthermore, we show that serum AAA are more frequently positive, with a significant association, in CD patients with total villous atrophy (77.8%), while are positive only in 22.2% of patients with subtotal IVA. Our results agree with those of other studies, namely that serum IgA-AAA positivity is indicative of histological damage to the intestine and especially of more severe histology damage. Nevertheless, percentage of positivity respect to intestinal atrophy is not always in agreement with other observations. Clemente et al. (10) reports, for retrospective study, that IgA-AAA were positive in 61.5% CD patients with total villous atrophy and in 41.4% CD patients with subtotal villous atrophy; Granito et al. (15) shows that half of CD patients with total or subtotal villous atrophy resulted positive for AAA, in contrast to 6% of those with mild intestinal mucosal damage. Carroccio et al. (22) reports that all patients with total or subtotal villous atrophy were positive for AAA, whereas patients negative for AAA had mild villous atrophy. Achour et al. (23) show that IgA-AAA was found in 32.7% of CD patients with total villous atrophy and in 9% of patients with subtotal villous atrophy, while Bazzigaluppi et al. (17) had values similar to our results (78.8% of positivity in patients with total villous atrophy, 27.5% of positivity in patients with subtotal villous atrophy). These differences of percentage could be due both to selection criteria (higher or lower prevalence of cases with severe mucosa damage) and to some technical bias related to methods used, about which we have just speculated.

The close link between the presence of AAA and the severity of tissue damage suggests that AAA could be secondary to intestinal mucosa damage in that it unmasks cryptic antigens. This phenomenon was further confirmed by the results we obtained for the a-tTG, which were the same for patients who were positive and negative for AAA: therefore, the relationship between AAA positivity and severity of damage seems not to depend on the immune response represented by the production of anti-tTG antibodies, but rather on the rapid disassembly of the actin network following damage to the intestinal mucosa (7, 8, 24).

The association of IgA-AAA with villous atrophy suggests a possible role for it in the noninvasive assessment of mucosal damage. Determination of IgA-AAA could be helpful in a-tTG and/or EMA-positive patients who cannot undergo an intestinal biopsy because of a severe contraindication or in the case of negative consensus regarding endoscopy. Moreover, the IgA-AAA assay could also furnish useful information when the clinician have to decide upon the advisability of performing a biopsy on certain categories of patients (children, patients already following a diet), or in the case of a histological picture that is difficult to interpret and may thus lead to diagnostic pitfalls (25,26). Major problems are usually related to patchy lesions, the poor sensitivity of endoscopic markers or artifactual damage caused by the difficulty of handling and/or correctly orienting biopsy tissues (25–28). Unfortunately, determination of IgA-AAA has also several limitations, besides the described advantages. As we have just observed, IgA-AAA assay is influenced by the performances of methods used, which show wide range of sensitivity and specificity; our results show that the method we used has high specificity and for this characteristic can be useful as test of second level, but it is fundamental to take into account serum storage conditions; moreover, IgA-AAA assays have not very high sensitivity and can certainly not take the place of much more sensitive tests such as a-tTG and EMA in the diagnosis of CD. We are in agreement with Fabbro et al. (16) who report that IgA-AAA assay has little to offer in diagnostic screening for CD compared to the well-established a-tTG assay. Nevertheless, the IgA-AAA positivity is strictly associated with severe mucosal damage, and they could be considered a further tool for the diagnosis of CD, given the mentioned advantages; moreover, in IgA-AAA-positive subjects, biopsies—and the costs inherent to them—could therefore be avoided.

In conclusion, the IFI commercial test that uses intestinal epithelial cells as substrate offers a useful method for IgA-AAA determination. Serum IgA-AAA positivity is indicative of more severe intestinal histology damage and their assay could be a real help to the clinician, especially in the a-tTG and/or EMA-positive complicated cases.

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