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Low Specificity of Anti-Tissue Transglutaminase Antibodies in Patients With Primary Biliary Cirrhosis

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The association between celiac disease (CD) and primary biliary cirrhosis (PBC) is well documented in medical literature; however, a high frequency of false positive results of the anti-transglutaminase (anti-tTG) test has been reported in patients with PBC. To verify if the positive results for anti-tTG autoantibody are false positives due to cross reactivity with mitochondrial antigens, we studied 105 adult patients affected with PBC, positive for anti-mitochondrial M2 antibodies. Anti-tTG IgA antibodies were studied by using six different immunoenzymatic assays that employ the tTG antigen obtained from different sources (human recombinant, placenta, red blood cells, and guinea pig liver). On the whole, 28 out of 105 PBC subjects tested positive for anti-tTG IgA antibodies, but only two were eventually found to be affected by CD; the other 26 were shown to be false positive. The specificity of the various antigenic substrates ranged from 88.5% of the human erythrocytes tTG to 97.1% of the human recombinant tTG. The results of this study showed that a true association between PBC and CD was present in only 2% of the patients and that, in most cases, the false positive results were attributable to the type of substrate utilized in the assay. J. Clin. Lab. Anal. 20:184–189, 2006.

Key words: celiac disease; tissue transglutaminase; mitochondrial antibodies; false positive

INTRODUCTION

The association between celiac disease (CD) and primary biliary cirrhosis (PBC), first described by Logan et al. (1), has also been described in various subsequent studies (2–14). At the same time, however, in subjects affected by PBC, numerous cases have been described as false positive for CD. This result has been linked to the poor specificity of the anti-gliadin antibody tests (15,16) and more recently, to a lesser extent, to the poor specificity of the tests used in the detection of anti-tissue transglutaminase (tTG) antibodies, particularly if the tTG extract of guinea pig liver is utilized (4,16–22). The test that utilizes human recombinant antigen has greatly reduced, but not completely eliminated, the incidence of false positives (23–28). Therefore, if in some cases of PBC the presence of anti-tTG antibodies has shown that there is a true coexistence of CD, in a far greater number of cases the positive result for anti-tTG has proved to be a false positive (29).

In a previous study, we analyzed the prevalence of anti-tTG antibodies in 618 subjects affected by various autoimmune diseases. A higher percentage of false positives was found in patients with PBC as compared to other autoimmune diseases: 10.4% vs. 1.6%,

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respectively (30). Because we used the more specific human recombinant antigen in that study rather than the guinea pig tTG, such a high percentage of positive results limited only to PBC seemed a possible expression of an antibody reactivity typically present in this disease, not an analytical false reactivity. Consequently, in this study, we wanted to verify if anti-tTG antibodies are actually present in higher concentrations in subjects affected by PCB than in patients with other autoimmune diseases, and also if this reactivity is CD-associated or if it pertains to a cross reaction with mitochondrial antigens, which are targets of PBC specific antibodies. We conducted the study using six different commercial methods for the class IgA antibodies and three methods for class IgG antibodies, using tTG antigen obtained from different sources so as to evaluate if the antibody reactivity was tied to the method and type of substrate used.

PATIENTS AND METHODS

A total of 105 adult patients with PBC were studied (91 women and 14 men; mean age, 63 years; range, 39–94). According to established criteria (31), the diagnosis of PBC was based on laboratory findings, the presence of anti-mitochondrial antibodies (AMA), and liver histology. All but one of the patients were positive for AMA IgG, as shown by indirect immunofluorescence technique on cryostatic sections of rat kidney (Inova, San Diego, CA) to a titer equal or greater than 1:80. In the 104 immunofluorescent positive sera, the AMA-M2 antibody specificity was confirmed by immunoblotting (Euroimmun, Lübeck, Germany) or by enzyme-linked immunosorbent assay (ELISA) (Pharmacia Diagnostics, Uppsala, Sweden). None of the 105 patients had clear symptoms of CD. All patients gave their oral consent to take part in the study.

Six different ELISA methods were used to determine the presence of anti-tTG antibodies of the IgA type. These assays utilized antigen from various sources including: two from human recombinant antigen (Eurospital, Trieste, Italy and Pharmacia), one from human placenta (Euroimmun), one from human erythrocytes (Inova) and two from guinea pig liver (Eurospital and Inova). Three ELISA kits were used for the research of class IgG antibodies; two utilized human recombinant antigen (Eurospital and Pharmacia) and one used tTG extracted from human red blood cells (Inova). The tests were performed in a single laboratory according to the manufacturers' instructions, including the indicated cutoff levels.

As a control group, 40 patients with CD and 40 healthy subjects were also tested with all the kits (with the exception of the human placenta kit). Sensitivity of the different kits in the CD group ranged from 97.5 to 100% and specificity in the healthy subject group ranged from 95.2 to 100%.

Using cryostatic sections of monkey esophagus (Eurospital), the sera that tested positive for anti-tTG antibody (IgA and/or IgG) were then tested for the presence of the more specific anti-endomysial antibody (EMA) of the IgA and/or IgG class. When the EMA test results were positive, patients were advised to undergo a biopsy of the small intestine in order to confirm the diagnosis of CD. In patients with positive anti-tTG and negative EMA, HLA DQα1*0501-DQβ1*0201 allele determination was performed (Protrans HLA Celiac Disease, Kesch, Germany), and those bearing this HLA phenotype, which is strictly associated with CD (32,33), were subjected to intestinal biopsy.

To verify if the positive results of the anti-tTG test were due to cross-reactivity between the anti-tTG and AMA antibodies, the AMA IgA in all of the sera was also measured using a immunoenzymatic assay (Inova). The study itself took into account that the anti-tTG positive sera would be adsorbed on mitochondrial antigens and then tested for anti-tTG. Nevertheless, several attempts to adsorb the anti-mitochondrial antibodies on M2 antigenic suspensions using pig heart, purified mitochondrial extract of monkey liver, and cryostatic sections of mouse kidney, proved to be unsuccessful. Indeed, even when the adsorption was carried out using sera that had been diluted 2,000 times, the anti-M2 reactivity continued to remain very high.

RESULTS

Overall, 28 sera out 105 (26.7%) were anti-tTG IgA positive and 6 (5.7%) anti-tTG IgG positive in at least one of the six ELISA methods used in the study. However, the agreement between all the ELISA kit results was very low: of the 28 IgA positive sera, seven showed reactivity with only one method, 12 with two methods, and two with three methods. Only three sera registered positive with five methods, and four with all six methods. All six anti-tTG IgG positive sera were positive with only one of the three tests used. Two sera out of 28 anti-tTG IgA positive were EMA IgA positive, and none of the six anti-tTG IgG positive were EMA IgG positive.

The HLA haplotype was determined in 24 of the 26 anti-tTG-positive EMA-negative patients, as two of them had since died. Five of the 24 were HLA positive; four underwent a duodenal biopsy (one refused further testing) and the histologic study showed a normal intestinal structure, excluding a CD diagnosis in all four patients. In the two EMA IgA positive patients, the subsequent biopsy showed the presence of diagnostic

TABLE 1. Number of the anti-tTG IgA- and IgG-positive sera and diagnostic specificity in PBC patients of the various ELISA methods employed*

tTG source	Human recombinant (Eurospital)	Human recombinant (Pharmacia)	Human placenta (Euroimmun)	Human red blood cells (Inova)	Guinea pig liver (Eurospital)	Guinea pig liver (Inova)	Positive cases any method
Anti-tTG IgA-positive	10	5	12	20	14	12	28
Anti-tTG IgG-positive	5	0	–	1	–	–	6
Specificity IgA (%)	92.2	97.1	90.3	82.5	88.3	90.3	
Specificity IgG (%)	95.1	100		99.0			

*The specificity was calculated for 103 patients, excluding the two celiac confirmed sera.

histology lesions for CD (sub-total villous atrophia and intraepithelial lymphocytosis, class 3a and 3b of the Marsh-Oberhuber classification) (34,35).

With the exception of the two patients with elevated anti-tTG IgA affected by CD, in almost all of the other samples the anti-tTG IgA antibody concentration was very close to the cut-off level. The best specificity was noted with the two methods that use human recombinant antigen (97.1 and 92.7%) and, to a lesser degree with the kit that uses tTG extracted from human placenta (90.3%), guinea pig liver (90.3 and 88.3%), and human erythrocytes (82.5%) (Table 1). On the other hand, the specificity of the tTG IgG test was far better than that of the anti-tTG IgA test, with 95.1 and 100% values for the two methods that use human recombinant tTG and 99% for the kit that uses human erythrocytes.

A positive AMA IgA reaction was observed in 25 samples (23.8%), 15 of which were also anti-tTG IgA positive with at least one ELISA method. In particular, the seven samples that were anti-tTG positive with the five to six ELISA methods all proved to be positive for AMA IgA, while 67 were negative for both antibodies, with an 82% correlation between the two tests (χ^2, 13.3; $P = 0.0003$).

DISCUSSION

Epidemiological studies have shown that CD is a disorder increasingly diagnosed in subjects that are asymptomatic or with sub-clinical progression (36). The discovery of these atypical cases was made possible by the availability of highly sensitive and specific immunological antibody tests. In particular, the recent clinical diagnostic introduction of the anti-tTG antibody test, which is far more specific than the anti-gliadin antibody test, and has in its human recombinant antigen formulation, a specificity almost equivalent to that of the EMA (28), has given rise to the question of whether the determination of the anti-tTG antibodies was sufficiently accurate so as not to require the use of other laboratory tests, to be used in conjunction with or as confirmation of the results. In a previous study, we had shown that the prevalence of CD in subjects with connective tissue disease or with gastrointestinal diseases was 0.3%, therefore not differing from that observed in the general population, and that the false positives were about 1.5%. On the other hand, in PBC affected patients, we noted that the false positives in the anti-tTG IgA test were about 10% (30). This is not new data since the elevated incidence of PBC false positives had already been shown in previous studies (4,17–19). However, all the previous studies used antigen extracted from guinea pig liver, possibly contaminated by other hepatic proteins that caused a highly reduced specificity, whereas we had used human recombinant antigens. The positives reactions we noted could be hypothetically attributed to two different possibilities: 1) the results were true positives, since some of the PBC subjects could have a low concentration of anti-tTG antibodies without suffering from CD or with a latent CD; and 2) the results were false positives due either to the type of antigen used in the test or to the existence of cross-reactivity between anti-mitochondrial antibodies and anti-tTG.

In order to verify these hypotheses, we conducted a study of a large number of patients with PBC and performed the anti-tTG IgA test with six different kits using four different antigenic sources: human recombinant antigens, antigens extracted from guinea pig liver, human placenta, and human erythrocytes. Of 105 subjects with PBC, 28 were anti-tTG IgA positive with at least one of the different methods used, but only two were subsequently found affected by CD (positive EMA and histological pattern indicative of CD). These findings confirmed that CD may be occasionally associated with PBC but, in patients affected by PBC, aspecific reactions from anti-tTG are very frequent and are mostly associated with the type of substrate used. In fact, of 26 anti-tTG IgA false positive sera, 21 only reacted with one to three out of six methods used, the least specific being the method utilizing antigen extracted from human erythrocytes (18% false positive)

and the most specific being those using human recombinant antigen (3–8% false positives). However, among the false positives, five out of 26 sera were positive with at least five out of six ELISA methods, showing that the positive result was not substrate-dependent. The majority of these sera showed antibody titers that were slightly higher than the cut-off, while the two anti-tTG IgA positive patients affected by CD had much higher levels of antibodies. Unfortunately, the difficulty in obtaining an adequate degree of adsorption, prevented us from verifying the possible presence of cross-reactivity between anti-tTG and anti-mitochondria antibodies.

In general, anti-tTG class IgA antibodies are considered a far more specific marker for CD than the anti-tTG IgG; it is interesting to note, however, that both in this study and in our previous one (30), many more false positives have been recorded for anti-tTG IgA than for anti-tTG IgG. This could possibly mean that anti-tTG IgA are physiologically produced in excess in some of the subjects with PBC. This raises the question as to why a considerable percentage of PBC patients has higher anti-tTG IgA concentrations compared to other autoimmune or hepatic diseases.

Primary biliary cirrhosis is a chronic autoimmune liver disease characterized by the presence of high-titer AMA and the progressive inflammatory destruction of intrahepatic bile ducts. The autoantigens recognized by AMA have been identified as members of the 2-oxo acid dehydrogenase (PDC-E2) enzyme family located in the inner mitochondrial membrane. However, several lines of evidence suggest that AMA are composed of a heterogeneous group of antibodies with differing specificities and that class IgA AMA (not IgG or IgM) perform a pathogenic function in PBC. In fact, it has been proven that the AMA serum IgA binds to the mitochondria only in subjects with PBC, not in control groups (37) and that, above all, the AMA IgA serum recognizes a PDC-E2 epitope that is different from the one recognized by AMA IgG and IgM (38).

Furthermore, although in PBC subjects the serum AMA is mostly of the IgM and IgG type (39), the biliary AMA is mostly of the IgA isotype (40,41) due to the presence in the ductal epithelium of IgA specific surface receptors that perform the IgA transcytosis (42). These data led to the hypothesis that PBC might develop after a locally AMA IgA driven response in the mucosa (43). We have found that AMA of the IgA isotype are present in the serum of 24% of PBC patients, and that there is a considerable correlation (82%) between the results of the AMA IgA and the anti-tTG IgA tests. The presence of high serum levels of anti-mitochondria IgA antibodies could explain the false positive reactions for IgA anti-tTG tests in patients with PBC observed in this and other studies. This could be due to the ease with which the IgA bind to the ELISA microwells, compared to other immunoglobulin classes, which is in accordance with what we have previously demonstrated in other studies (44,45).

In conclusion, this study shows an association between PBC and CD in 2% of the cases, a slightly higher percentage, though not very different from that observed in screening studies or in patients with other autoimmune or chronic liver diseases (46,47). In addition, we have shown that a significant percentage of subjects with PBC may prove falsely positive to the tests looking for anti-tTG antibodies, and that in the majority of cases these false positives were attributable to the type of substrate used. Furthermore, this study proves that there is, at present, no antigen source able to guarantee a specificity of 100%, as false positives may occur with any antigen used.

Finally, even if it has been shown that the EMA probably recognize the same antigen target of the anti-tTG antibodies, with this group of patients the EMA IgA test has proved to be completely specific, being positive only in the two subjects affected by both PBC and CD. As a consequence, it is important that in PBC patients (and, in general, in all cases with positive results of anti-tTG) positive findings in the anti-tTG antibody assay are confirmed by the EMA test. In the case of negative EMA results, it is only after clinical evaluation that it may be decided if it is to proceed with further studies (HLA, intestinal biopsy) in order to exclude the contextual presence of CD.

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