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Are Antibodies to Carbonic Anhydrase II Specific for Anti-Mitochondrial Antibody-Negative Primary Biliary Cirrhosis?

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Antibodies to carbonic anhydrase II (CAII) have been reported to be specific to anti-mitochondrial antibody (AMA) -negative primary biliary cirrhosis (PBC). We examined whether antibodies to CAII are specific for AMA-negative PBC or a nonspecific response in autoimmune liver disease. Antibody assays to CAII, by western immunoblot (dilution 1:200), were performed on sera from 16 AMA-negative PBC patients, 21 AMA-positive PBC patients, 21 autoimmune hepatitis type 1 (AIH) patients, and 18 alcoholic liver disease (ALD) patients. CAII antibody activity was found in 8 of 16 (50%) of the AMA-negative PBC patients, 9 of 21 (43%) of the AMA-positive PBC group, 10 of 21 (48%) of the AIH group, and in 3 of 18 (17%) of the ALD control group. There was no difference in the prevalence of CAII antibody reactivity between the AMA-negative PBC, AMA-positive PBC, and AIH groups. In conclusion, we determined that CAII antibodies are detected with equal frequency in AMA-positive PBC and AIH. Given that CAII antibodies have been reported in other nonhepatic autoimmune diseases, we conclude that CAII antibodies are likely a nonspecific marker of autoimmunity rather than specific for AMA-negative PBC.

KEY WORDS: primary biliary cirrhosis; anti-mitochondrial antibody; autoimmune liver disease; carbonic anhydrase II antibodies.

Anti-mitochondrial antibody (AMA) -negative primary biliary cirrhosis (PBC) is clinically, biochemically, and histologically indistinguishable from AMA-positive PBC. It is unclear whether these patients represent a subset of PBC (1, 2), autoimmune hepatitis (AIH (3), or are a distinct entity unto themselves (4, 5).

Antibodies to CAII, a zinc metal enzyme found in biliary epithelial cells (6), have been described as specific to AMA-negative PBC (7). CAII antibodies have been reported in other nonhepatic autoimmune diseases, such as SLE, scleroderma, Sjögren's syndrome, polymyositis, idiopathic chronic pancreatitis, and endometriosis (8–15).

In this study, we examined a patient population with different autoimmune liver diseases, using patients with alcoholic liver disease as controls, to establish if antibodies to CAII are disease-specific for AMA-negative PBC or a non-specific response found in autoimmune liver disease.

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MATERIALS AND METHODS

Study Patients. The subjects were recruited from an outpatient liver clinic in a tertiary referral center in Toronto, Canada. Sixteen AMA-negative PBC patients, diagnosed on the basis of cholestatic liver biochemistry and a compatible liver biopsy, were found to test negative for

TABLE 1. AMA-NEGATIVE PBC PATIENT CHARACTERISTICS*

Patient	Age (yr)	Gender	Total bilirubin (μmol/liter)	ALP (unit/liter)	ALT (unit/liter)	AMA-M2 (IU/ml)	ANA	SMA
1. J.B.	66	F	86	723	88	4	+	+
2. J.C.	51	F	7	270	107	4	+	+
3. Y.C.	64	F	8	291	47	5	+	−
4. O.D.	66	F	5	101	39	3	+	+
5. W.D.	37	F	45	1111	171	4	+	−
6. S.E.	79	F	129	493	33	N/A†	+	+
7. K.F.	66	F	33	245	35	9	+	+
8. M.G.	78	F	101	621	N/A	4	−	+
9. R.G.	70	F	14	199	45	3	−	+
10. L.H.	52	M	28	903	117	2	+	+
11. M.I.	70	F	9	112	20	3	+	−
12. K.K.	53	F	6	352	131	3	+	−
13. D.L.	45	F	18	1885	118	2	−	+
14. C.M.	48	M	40	970	208	3	+	+
15. C.P.	58	F	8	156	35	2	+	+
16. W.P.	44	F	15	335	45	5	+	−

*Normal values: total bilirubin <20 μmol/liter; ALP <100 unit/liter; ALT <40 unit/liter. AMA-M2 ELISA: negative <10 IU/ml.
†See text for details.

AMA by indirect immunofluorescence and confirmed with ELISA for antibodies against the M2 component of the mitochondrial inner membrane. They had not received any specific therapy at the time of study. For comparison, two other autoimmune liver disease groups, AMA-positive PBC and autoimmune hepatitis type 1 (AIH), were selected. Twenty-one of these were patients who had elevated serum alkaline phosphatase levels, were AMA-positive by indirect immunofluorescence, and had histological evidence consistent with PBC. They were not on any specific therapy at the time of study. Twenty-one patients with chronic (nonviral) hepatitis with elevated IgG levels and who were ANA- and/or SMA-positive and had liver biopsies consistent with AIH were also included in this study; most of them were receiving immunosuppressive therapy and were in remission. Eighteen patients with a history of excess alcohol intake and histological evidence of alcoholic liver injury (ALD) were chosen as controls. The presence of other liver disease was ruled out where appropriate. Patient charts were reviewed for clinical features, liver biochemistry, serology for ANA and SMA, and immunoglobulin quantification.

Serological Testing for CAII Antibodies. Serum was tested for CAII antibodies by western immunoblotting (dilution, 1:200). All necessary reagents and equipment were purchased from Novex (San Diego, California). The protocols of the manufacturer were followed throughout. Briefly, CAII was purchased from Sigma Chemicals (St. Louis, Missouri) and was electrophoresed under reducing conditions with mercaptoethanol on 12% 2D gradient polyacrylamide gels. It was then electrotransferred to Hybond-ECL nitrocellulose membranes (Amersham International, Arlington Heights, Illinois). The membranes were blocked overnight and further processed according to manufacturer recommendations. The membranes were divided into 10 equal strips for a CAII concentration of 10 ng/strip. Serum for testing was diluted 1:200 in 6% BSA containing 0.05% Tween 20. Diluted serum was incubated with a strip for 3 hr and washed for 1 hr with western blot wash solution (Tris base 12 gs, NaCl 40 gs, 15 ml Tween 20 in 5 liters, pH 7.6). Furthermore, the strips were incubated for 1 hr in diluted (1:2000) horseradish peroxidase-conjugated secondary anti-human antibody and further washed for 1 hr as above. Detection of enzyme activity was by chemiluminescence and captured on x-ray film. Biotinylated markers were visualized with streptavidin horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 6% BSA.

Statistical Analysis. Differences in the proportion of AMA-negative PBC, AMA-positive PBC, AIH, and ALD patients testing positive for CAII antibodies were compared by means of 95% confidence intervals and χ^2 or Fisher's exact tests using SAS (SAS Institute, Cary, North Carolina).

RESULTS

Sixteen patients, who by all other criteria had PBC, tested negative for AMA by immunofluorescence (IF) and were confirmed to be negative by ELISA for the M2 mitochondrial component (Table 1). One patient, S.E., was AMA-negative by IF but died before confirmatory ELISA testing could be performed. Eighty-eight percent (14 of 16) were female with a mean age of 59 at the time of study. All had an abnormal alkaline phosphatase value (median 548 units/liter, range 101–1885) and nine had elevated ALT values (median 47 units/liter, range 20–208). The mean AMA-M2 ELISA value was 3.7 (negative < 10 IU/ml). Thirteen were positive for ANA and 11 for SMA.

Twenty-one PBC patients (mean age 57, 90% female), 21 AIH patients (mean age 45, 71% female), and 18 ALD patients (mean age 56, 73% male) participated in the study. Clinical, biochemical and sero-

TABLE 2. SUMMARY OF PATIENT GROUP CHARACTERISTICS*

Group	*Age [mean (range)]*	*Gender (% F)*	*ALP [median (range)]*	*ALT [median (range)]*
AMA-negative PBC (N = 16)	59 (37–79)	89	344 (101–1885)	47 (20–208)
AMA-positive PBC (N = 21)	57 (32–78)	90	515 (106–1127)	87 (18–297)
AIH (N = 11)	45 (21–75)	71	81 (38–161)	35 (4–519)
ALD (N = 18)	57 (29–71)	73	96 (52–214)	19 (9–38)

*Normal values: total bilirubin <20 μ mol/liter; ALP <100 unit/liter; ALT <40 u/liter.

logical parameters obtained at the time of study are summarized in Table 2.

Eight of 16 (50%) AMA-negative PBC patients tested positive for CAII antibodies (Table 3). The prevalence of CAII antibodies in the AMA-positive PBC and AIH patients was similar, 9 of 21 (43%) and 10 of 21 (48%), respectively. Three of 18 (17%) ALD control patients tested positive for CAII antibodies. There was no difference in the prevalence of CAII antibody reactivity between the AMA-negative PBC, AMA-positive PBC, and AIH groups. The 33% difference obtained in CAII antibody reactivity between the AMA-negative PBC group (50%) and the ALD control group (17%) lies within the 95% confidence interval (3.4–63.3).

DISCUSSION

Antibodies to CAII were first described in autoimmune liver disease in a study by Gordon et al (7). They found CAII antibody reactivity in 4 of 5 patients with AMA-negative PBC, 1 of 12 with AMA-positive PBC, 1 of 12 with AIH and in none of 8 Gilbert syndrome control patients. They concluded that antibodies to CAII were disease-specific for AMA-negative PBC. They speculated that the antibody was probably an immune reaction to CAII exposure from bile duct injury and, thus, was unlikely to play a primary pathogenic role.

Muratori et al (16) were unable to validate the findings of Gordon et al (7). Using protocols reported by Gordon et al (7), they found CAII antibodies in the sera of 2 of 2 (100%) AMA-negative PBC patients, 9 of 20 (45%) AMA-positive PBC patients, 3 of 7 (57%) AIH patients, and 10 of 20 (50%) healthy blood donor controls. A similar distribution of reactivity, with lower overall prevalence, was noted when serum was diluted further from 1:40 to 1:200. Given the high prevalence of CAII antibodies in other autoimmune liver diseases and in healthy controls, they were unable to support the disease-specificity of CAII antibodies.

A recent study by Invernizzi et al (17) determined the prevalence of CAII antibodies in AMA-negative PBC, AMA-positive PBC, AIH, nonhepatic autoimmune disease, and healthy subjects. The overall prevalence of CAII antibodies in PBC sera, determined in 215 consecutive patients, was 8.0% using a dilution of 1:100. Their results showed that CAII antibody reactivity was not related to AMA status in PBC patients. They failed to detect CAII antibody reactivity in their patients with AIH and healthy controls.

Using protocols and positive controls supplied by Dr. Gordon, we detected CAII antibody activity with equal prevalence in AMA-negative and -positive PBC patients (50% and 43%). In addition, CAII antibody activity was detected in 48% of AIH patients. Carbonic anhydrase antibodies have been reported in other nonhepatic autoimmune diseases, SLE, scleroderma, Sjögren's syndrome, polymyositis, idiopathic chronic pancreatitis, and endometriosis (8–15). Antibody titers have been shown, in some, to parallel disease activity (11); however, their pathophysiologic role in autoimmune disease, if any, is unknown.

Patients with ALD may also have autoimmune

TABLE 3. SUMMARY OF CAII ANTIBODY ACTIVITY

*CAII antibody activity**	*AMA-negative PBC (N = 16)*	*AMA-positive PBC (N = 21)*	*AIH (N = 21)*	*ALD (N = 18)*
Positive	8	9	10	3
Negative	8	12	11	15

*Positive results detected at a dilution of 1:200.

features, such as ANA reactivity (18, 19) and an immune reactivity to Mallory's hyaline (20, 21). The finding of CAII antibodies in ALD patients is perhaps a further manifestation of a heightened immune response in such patients.

We determined that CAII antibodies are commonly detected in patients with autoimmune liver disease. We conclude that CAII antibodies are likely a nonspecific marker of autoimmunity rather than specific for AMA-negative PBC. The presence of CAII antibodies in other autoimmune diseases supports this conclusion (8–15).

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