Serial Changes in Enzyme Inhibitory Antibody to Pyruvate Dehydrogenase Complex During the Course of Primary Biliary Cirrhosis

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To assess the usefulness of enzyme inhibition assay for the diagnosis of primary biliary cirrhosis (PBC), we determined the serial changes in enzymatic inhibitory antibody to pyruvate dehydrogenase complex (PDC) in patients with PBC, and compared the results to those of immunofluorescence and immunoblotting. Forty-nine sera from 19 patients with PBC who were followed-up for at least 16 months were tested for antimitochondrial antibodies (AMA) by indirect immunofluorescence, immunoblotting on bovine heart mitochondria, and enzyme inhibition assay using commercially available TRACE Enzymatic Mitochondrial Antibody (M2) Assay (EMA) kit. Of the 49 sera, 39 (80%), 35 (71%), 38 (78%), 31 (63%), and 36 (73%) were positive for AMA by immunofluorescence, for immunoglobulin G (IgG), IgM, and IgA class antibody against E2 subunit of PDC (PDC-E2) by immunoblotting, and for enzymatic inhibitory antibody to PDC by EMA, respectively. AMA titers determined by immunofluorescence did not change in 9 patients (47%), increased in 4 (21%), decreased in 3 (16%), and fluctuated in 3 (16%)

during follow-up. The number of anti-M2 bands by immunoblotting did not change in 9 (47%), increased in 6 (32%), decreased in 2 (11%), and fluctuated in 2 (11%). Units of PDC activity by EMA did not change markedly in 16 (84%), increased in 2 (11%), and fluctuated in 1 (5%). Positive EMA results were common in cases with high levels of serum alkaline phosphatase and IgM, and the units of PDC activity by EMA correlated significantly and inversely with AMA titers by immunofluorescence, and serum reactivity to PDC-E2 by immunoblotting, respectively. There was no correlation between serial changes in biochemical data and units of PDC activity by EMA. In three patients who showed a decrease in AMA titers, AMA titers correlated more with EMA results than immunoblotting. Moreover, in a patient with fluctuating AMA titers, the units of PDC activity by EMA paralleled AMA titers. Our results suggest that EMA is useful for the diagnosis of AMA-positive PBC, and also could be used for monitoring the disease course in PBC. *J. Clin. Lab. Anal.* 14:208–213, 2000. © 2000 Wiley-Liss, Inc.

Key words: primary biliary cirrhosis; antimitochondrial antibody; enzyme inhibition assay; pyruvate dehydrogenase complex

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease associated with damage of small and medium-sized bile ducts, which leads to cholestasis and progressive fibrosis. The cause of the disease is unknown but is thought to be related to certain inherited disorders of immunoregulation. Antimitochondrial antibodies (AMA) detected by indirect immunofluorescence are found in 95% of patients with PBC, and they have a specificity of more than 98% for this disease (1,2). This test can detect reactivity to all 2-oxo-acid dehy-

drogenase complex (2-OADC) enzymes, but is nonautomated and its “read-out” is subjective.

Recently, new serological assays have been developed for the detection of anti-M2 antibodies including immunoblotting, enzyme-linked immunosorbent assay (ELISA), and enzyme

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Received 2 February 2000; Accepted 20 March 2000

inhibition assay (2). Of these, enzyme inhibition assay, which measures the capacity of PBC sera to inhibit the catalytic activity of pyruvate dehydrogenase complex (PDC), is nonsubjective compared to immunofluorescence, is more rapid and technically simpler than immunoblotting and ELISA, and has a sensitivity and specificity comparable to other assays (3–6). Therefore, this assay could be particularly applicable to population screening for early stages of PBC (6). However, the serial changes in the inhibitory activity of PBC sera to PDC during the course of the disease have not been fully assessed (4,7). In the present study, we investigated the serial changes in enzymatic inhibitory antibody to PDC during the course of PBC to assess the advantages of enzyme inhibition assay for the diagnosis of this disease.

MATERIALS AND METHODS

Patients

Forty-nine serum samples were collected from 19 patients with PBC (18 females and 1 male, aged 42–77 years; mean 56 years at the time of diagnosis) who were followed-up for at least 16 months (mean observation period, 31 months; range, 16–45 months). All patients fulfilled the specified clinical and/or histological diagnostic criteria of PBC (1,8–10), and all were being treated during the period of serum sampling with 300–600 mg/day of ursodeoxycholic acid (UDCA). Liver biopsy was performed in 17 patients (89%) and according to the Scheuer's criteria (9), the stage of PBC was I in 8, II in 3, III in 4, and IV in 2. Ten (53%) patients were asymptomatic. Sera were tested for levels of total bilirubin (T. Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (γ -GTP), total cholesterol (T. Chol), and immunoglobulin M (IgM). The serial serum samples, which were obtained after informed consent, had been stored at -80°C until the assay.

AMA by Indirect Immunofluorescence

AMA was tested by indirect immunofluorescence using cryostat sections from rat kidney and stomach with antihuman γ -globulin conjugated with fluorescein isothiocyanate (FITC) as the second antibody. A serum titer of 1:20 or greater was defined as a positive reaction.

Immunoreactivity Against 2-OADC by Immunoblotting

Sera were tested by immunoblotting at a dilution of 1:5,000 for detection of IgG antibodies, 1:1,000 for IgM antibodies, and 1:1,000 for IgA antibodies against bovine heart mitochondria (BHM) prepared by differential centrifugation from homogenized bovine heart (11), kindly provided by Dr. Ian R. Mackay (Monash University, Australia) as previously described (12). Peroxidase-coupled affinity-purified goat anti-

human IgG (H+L) (Bio-Rad, Richmond, CA), IgM, and IgA (Zymed Laboratories, San Francisco, CA) were used as the second antibody, and enhanced chemiluminescence (ECL) Western-blotting detection reagents (Amersham Inc., Buckinghamshire, UK) as the peroxidase substrate. The 74-, 52-, 50-, 46-, and 41-kDa components of BHM represented the E2 subunit of PDC (PDC-E2), protein X (E3 binding protein), the E2 subunit of branched chain oxo-acid dehydrogenase complex (BCOADC-E2), the E2 subunit of 2-oxoglutarate dehydrogenase complex (OGDC-E2), and E1 α subunit of PDC (PDC-E1 α), respectively (12). Nonreactive sera were retested at a dilution of 1:500 for detection of IgG antibodies, and 1:100 for IgM and IgA antibodies before being labeled as negative. Two standard sera from patients with PBC, known to be reactive with all of the above five components of 2-OADC served as the positive control. The sensitivity and specificity in our assay condition were 99 and 86%, respectively (12).

Pyruvate Dehydrogenase Complex Enzyme Inhibitory Antibodies by Enzyme Inhibition Assay

The enzyme inhibition assay was performed using the commercially available TRACE Enzymatic Mitochondrial Antibody (M2) Assay (EMA) (TRACE Scientific, Victoria, Australia) kit. This kit is based on the evidence that the principal anti-PDC antibody inhibits the catalytic activity of the PDC complex in vitro. The assay was performed as follows. The "substrate reagent" (250 μL) containing 0.6 mmol/L sodium pyruvate, 0.5 mmol/L magnesium acetate, 0.1 mmol/L cocarboxylase, 0.065 mmol/L coenzyme A, and 1.25 mmol/L nicotinamide adenine dinucleotide (NAD), was placed in flat-bottomed microtiter wells (Nunc-Immuno Plate, Roskilde, Denmark). Thereafter, 4 μL of undiluted test serum was added to each well and incubated for 1 min at 37°C before adding 50 μL of the "enzyme reagent" containing $> 300 \text{ U/L}$ pyruvate dehydrogenase and 10 mmol/L dithiothreitol. After 30 sec of lag time, the rate of reaction was monitored by measuring the rate of increase in absorbance at 340 nm in a microplate reader. The reaction rate (RR) was calculated using the absorbance values at 0 and 2 min based on the following formula: (final absorbance – initial absorbance)/time. The units of activity (%) was derived from the formula: (test RR/standard RR) $\times$ 100. The standard RR was derived from the "calibrator" wells that contained anti-PDC antibody-free serum (100% activity). The unit of PDC activity of less than 70% was considered anti-PDC positive with sensitivity and specificity of 82% and 100%, respectively, based on the information provided by the manufacturer. To validate our assay condition, 50 sera from other than PBC (20 normal healthy subjects, 21 patients with histologically proven chronic hepatitis B or C, 6 with fatty liver, 2 with drug-induced hepatitis, and 1 with alcoholic liver cirrhosis) were also tested. Using a selected positive serum with a moderate

degree of inhibitory activity from a patient with PBC, replicate testing for intra-assay variation ($n = 8$) yielded a mean (SD) unit of activity of 77.6% (4.2%) with a coefficient of variation (CV) of 5.4%, and replicate testing for inter-assay variation ($n = 10$) gave a mean (SD) units of activity of 73.3% (2.4%) with a CV of 3.3%.

Statistical Analysis

Values were expressed as mean $\pm$ SD. Differences between groups were examined for statistical significance using the two-tailed Kruskal–Wallis test, chi-square test, or Fisher's exact probability test, using Statview software, version J-4.5 (Abacus Concepts, Berkeley, CA) on a Macintosh computer. A P value less than 0.05 was considered significant.

RESULTS

Reactivity to PDC in Different Assays

By immunofluorescence, 39 (80%) of the 49 PBC sera were positive for AMA (AMA-positive). The AMA titers were 1:20 in 2 positive cases, 1:40 in 5, 1:80 in 10, 1:160 in 6, and 1:320 in 16. By immunoblotting, 48 (98%) had IgG, IgM, or IgA class antibodies against at least one of the 2-OADC enzymes. Of these, 8 were positive for IgG, IgM, or IgA class antibodies against other than 74-kDa components (PDC-E2). Therefore, 40 (82%) sera were positive for IgG, IgM, or IgA class antibodies against PDC-E2. Of these, five sera reacted very weakly to this component (Fig. 1). By EMA, 36 (73%) inhibited the catalytic activity of the PDC enzyme (units of PDC activity less than 70%) (Table 1). On the other hand, none of 50 sera from patients with chronic hepatitis B or C, fatty liver, drug-induced hepatitis, alcoholic liver cirrhosis, and normal healthy subjects inhibited the catalytic activity of this enzyme.

Biochemical Laboratory Data and Immunological Parameters

Sera with elevated serum levels of T. Chol. were less common ($P < 0.01$), and those showing elevated IgM were more frequent ($P < 0.05$) in AMA-positive cases by immunofluorescence. Sera with elevated ALP and IgM were also more common in anti-PDC antibody-positive (units of PDC activity less than 70%) cases by EMA, respectively ($P < 0.05$). There was no significant correlation between liver function tests and the results of anti-PDC-E2 antibody by immunoblotting (Table 2).

Correlation Between AMA Titers by Immunofluorescence and Units of PDC Activity by EMA

All ten AMA-negative sera failed to inhibit the catalytic activity of the PDC enzyme by EMA. Conversely, 36 (92%)

Fig. 1. Representative immunoblots from preparation of bovine heart mitochondria probed with PBC sera. Sera were used at a dilution of 1:1,000. Positive control PBC serum with known IgG-class reactivity with 2-OADC enzymes at molecular weights (M.W.) of 74 kDa (PDC-E2), 52 kDa (protein X), 50 kDa (BCOADC-E2), and 46 kDa (OGDC-E2) is shown in lane 1. The serum reacted strongly with a protein of 74 kDa and moderately with a protein of 52 kDa as shown in lane 2. The serum reacted very weakly to 74-kDa protein as shown in lane 3. A nonreactive PBC serum is shown in lane 4.

of 39 AMA-positive sera inhibited the activity of the enzyme. There was a significant inverse correlation between AMA titers by immunofluorescence and units of PDC activity by EMA ($P < 0.01$, Fig. 2). Of the three AMA-positive sera that did not inhibit the catalytic activity of the PDC enzyme by EMA, two had IgG class antibody exclusively to BCOADC-E2 but not PDC-E2, whereas another one was positive for all IgG, IgM, and IgA class antibodies to PDC-E2.

Correlation Between IgG, IgM, and IgA Class Anti-PDC-E2 by Immunoblotting and Units of PDC Activity by EMA

Eight (89%) of 9 sera negative for IgG, IgM, and IgA class antibodies to PDC-E2 by immunoblotting did not inhibit the catalytic activity of the PDC enzyme by EMA, whereas 34 (97%) of 35 sera positive for these antibodies inhibited the activity of the enzyme. However, four of five sera that reacted very weakly to PDC-E2 by immunoblotting did not inhibit the activity of the enzyme. Overall, the mean value of

TABLE 1. Reactivity to PDC in patients with PBC in different assays^a

AMA by IF	74 kDa (PDC-E2) by immunoblotting			EMA	No. of sera
	IgG	IgM	IgA		
–	–	–	–	–	6
–	w	–	–	–	2
–	–	w	–	–	2
+	–	–	–	–	2
+	–	–	–	+	1
+	–	w	–	+	1
+	–	+	–	+	2
+	w	+	w	–	1
+	+	+	–	+	2
+	+	+	+	+	30
39	35	38	31	36	49

^aPDC, pyruvate dehydrogenase complex; PBC, primary biliary cirrhosis; AMA, antimitochondrial antibody; IF, immunofluorescence; EMA, TRACE Enzymatic Mitochondrial Antibody (M2) Assay; –, negative; +, positive; w, weakly positive.

the units of PDC activity by EMA was significantly lower in sera with anti-PDC-E2-positive than in those with negative, or weakly positive, respectively ($P < 0.01$) (Fig. 3). The clinical background of patients in whom the serum samples showed discrepancy between immunoblotting and EMA is summarized in Table 3. Serum numbers 1 and 2, and 4 and 5 were from the same patients, respectively.

Serial Changes of Biochemical Laboratory Data and Immunological Parameters

During the course of follow-up, the titer of AMA by immunofluorescence did not change in 9 patients (47%), increased in 4 (21%), decreased in 3 (16%), and fluctuated in 3 (16%). The number of anti-M2 bands by immunoblotting did not change in 9 (47%), increased in 6 (32%), decreased in 2 (11%), and fluctuated in 2 (11%). The units of PDC activity by EMA did not change markedly in 16 (84%), increased in 2 (11%), and fluctuated in 1 (5%). There were no correlations between serial changes in each biochemical laboratory test

and immunological parameters (data not shown). The units of PDC activity by EMA did not vary significantly in all nine patients in whom the titer of AMAs did not change during the course of follow-up. However, the number of anti-M2 bands by immunoblotting changed in four of these patients (two increased and two decreased). The units of PDC activity decreased in all four patients whose AMA titers had increased, but the number of anti-M2 bands increased in three patients, and did not change in one patient. The units of PDC activity increased in all three patients in whom AMA titers had decreased, but the number of anti-M2 bands in these patients did not decrease (one increased and two did not change). Of the 3 patients in whom AMA titers showed some fluctuation, the units of PDC activity by EMA (from 57 to 96%) paralleled the AMA titers in 1 patient.

DISCUSSION

A “miniaturized” semiautomated enzyme inhibition assay was recently developed for the detection of anti-M2 antibodies (3). Several studies have assessed its objectivity, rapidity, simplicity, and cost effectiveness, by comparing these parameters to other assays such as immunofluorescence, immunoblotting, and ELISA (2,4,6,13). Schmit et al. (14) tested 23 sera from patients with AMA-positive PBC and 92 from non-PBC including healthy controls using a new commercially available EMA supplied by TRACE Scientific, and compared the results to those of immunofluorescence and in-house ELISA. They reported that the sensitivity and specificity of EMA were quite sufficient compared to other assays. Our present results indicated that the sensitivity and specificity of EMA were 73 and > 98%, respectively. The lower sensitivity in our assay condition may be explained by the fact that EMA can detect the inhibitory activity of sera to PDC but not to the other 2-OADC enzymes such as BCOADC or OGDC. The lower sensitivity also may be due to the relatively high frequency of AMA-negative (by immunofluorescence) PBC patients in our study subjects. Our results also indicated that the units of PDC activity by EMA correlated significantly and inversely with AMA titers by immunofluorescence and

TABLE 2. Biochemical laboratory data and immunological parameters^a

Laboratory test	No. tested	Total	IF		Immunoblotting		EMA	
			Negative	Positive	Negative	Positive	Negative	Positive
T. Bil	49	12/37	1/9	11/28	2/7	10/30	2/11	10/26
AST	49	19/30	2/8	17/22	2/7	17/23	2/11	17/19
ALT	49	14/35	2/8	12/27	2/7	12/28	2/11	12/24
ALP	49	34/15	6/4	28/11	4/5	30/10	6/7 ^b	28/8 ^b
γ-GTP	49	26/23	5/5	21/18	3/6	23/17	5/8	21/15
T. Chol	49	10/39	5/5 ^c	5/34 ^c	4/5	6/34	5/8	5/31
IgM	46	28/18	2/7 ^b	26/11 ^b	3/5	25/13	4/8 ^b	24/10 ^b

^aData represent the number of cases with elevated level/normal level. IF, immunofluorescence; EMA, TRACE Enzymatic Mitochondrial Antibody (M2) Assay.

^b $P < 0.05$.

^c $P < 0.01$.

Fig. 2. Correlation between AMA titers by immunofluorescence and units of PDC activity by EMA. The mean ($\pm$ SD) unit of PDC activity by EMA were $84 \pm 10\%$ in AMA-negative sera ($n = 10$), 65% in sera with an AMA titer of 1:20 ($n = 2$), $35 \pm 31\%$ in 1:40 ($n = 5$), $30 \pm 24\%$ in 1:80 ($n = 10$), $17 \pm 15\%$ in 1:160 ($n = 6$), and $4 \pm 2\%$ in 1:320 ($n = 16$). There was a significant inverse correlation between AMA titers and units of PDC activity by EMA. PDC, pyruvate dehydrogenase complex; EMA, TRACE Enzymatic Mitochondrial Antibody (M2) Assay; AMA, antimitochondrial antibody.

serum reactivity to PDC-E2 by immunoblotting, respectively, although the proportion of positive anti-PDC antibody by EMA was slightly lower than that by immunofluorescence and immunoblotting. Moreover, positive EMA results were common in cases with elevated serum levels of ALP and IgM, which are the characteristic biochemical parameters in PBC (8). These results indicated that EMA was useful for the diagnosis of AMA-positive PBC.

It is well known that in PBC the AMA titers measured by immunofluorescence often fluctuate considerably even from a negative result to a positive one, and vice versa, during the course of the disease, with or without treatment (15). Furthermore, AMA titers do not correlate with disease progression, and there is no evidence that a decrease in AMA titer is associated with a therapeutic response (16,17). Several reports have recently described the serial changes in serum reactivity in other immunological tests such as immunoblotting, ELISA, and enzyme inhibitory assay during the course of the

Fig. 3. Correlation between IgG, IgM, and IgA class anti-PDC-E2 by immunoblotting and units of PDC activity by EMA. The mean ($\pm$ SD) unit of PDC activity by EMA was $76 \pm 5\%$ in anti-PDC-E2-negative sera ($n = 9$), $81 \pm 26\%$ in anti-PDC-E2 weakly positive sera ($n = 5$), and $15 \pm 18\%$ in anti-PDC-E2 strongly positive sera ($n = 35$) by immunoblotting. The mean value of the units of PDC activity by EMA was significantly lower in sera positive for anti-PDC-E2 than in negative samples, or weakly positive ($P < 0.01$). PDC, pyruvate dehydrogenase complex; EMA, TRACE Enzymatic Mitochondrial Antibody (M2) Assay.

disease. Teoh et al. (4) demonstrated that the titers of antibodies to PDC by ELISA and enzyme inhibition assay were significantly lower but the reactivity by immunoblotting remained unchanged after 12 and 24 months of cyclosporine treatment. Kisand et al. (7) also reported that two-year UDCA treatment resulted in a decrease of the serum level of antibodies to PDC by ELISA, and also a reduction of the inhibitory activity of PBC sera to PDC by enzyme inhibition assay, but did not influence the immunoblotting pattern. Furthermore, Miyakawa et al. (18) reported that by immunoblotting the anti-M2 antibodies did not change in most of 21 PBC patients who were followed-up for an average of 60.7 months. These data suggest that a close serological monitoring of the level of autoantibody by ELISA or enzyme inhibition assay (but not immunoblotting) may be informative for evaluating the disease course of PBC. In the present study, however, we

TABLE 3. Clinical background of patients showing discrepancy between the results of immunoblotting and EMA^a

Patient	Serum no.	Age (year)	Sex	Scheuer's stage ^b	Symptom	AMA by IF (titer)	Immunoblotting (kDa) ^c			EMA (%)
							IgG	IgM	IgA	
1	1	43	F	1	A	1:40	46	—	—	67.5
1	2	46	F		A	—	—	w74	—	96
2	3	61	F	3	S2	—	50	w74	—	79
3	4	60	F	1	S1	—	w74	52	—	106
3	5	62	F		S1	—	w74	52	—	85.5
4	6	53	F	2	S1	1:20	w74/w52	74/w52/w50	w74	73

^aEMA, TRACE Enzymatic Mitochondrial Antibody (M2) Assay; AMA, antimitochondrial antibody; IF, immunofluorescence; F, female; A, asymptomatic; S1, pruritus; S2, jaundice; —, negative; w, weakly positive; 2-OADC, 2-oxo-acid dehydrogenase complex.

^bScheuer's staging of 1–4 for PBC (9).

^cFigure indicates the molecular weight (kDa) of each 2-OADC enzyme reacted with serum, as shown in Materials and Methods.

could not find any correlation between serial changes in any biochemical laboratory parameter and the units of PDC activity determined by EMA. This may be due, in part, to the limited number of patients and relatively high frequency of AMA-negative (by immunofluorescence) PBC patients in our study subjects. In particular, AMA titers correlated more with EMA results than immunoblotting in three patients whose AMA titers decreased in the present study. Moreover, in one patient who demonstrated fluctuation of AMA titers, the units of PDC activity by EMA paralleled the AMA titers. These data suggest that disease course of PBC may influence the EMA results.

In conclusion, the present study demonstrated that EMA is useful for the diagnosis of AMA-positive PBC and could be used to monitor the disease course of PBC, particularly due to the small amount of serum requested (only 4 μ L of undiluted test serum), objective read-out, and rapid turnaround time (approximately 6 min for 10 serum samples).

ACKNOWLEDGMENTS

The authors thank Drs. Hajime Tanioka, Kenji Hayashida, Masahiro Senju, Saburo Shikuwa, Mitsuru Kawamoto, Kazuyuki Imamura, Yoshihiko Umene, Shun-ichi Morikawa, Kohei Komatsu, Michio Tomonaga, Kenji Shirono, Ichiei Egawa, Hirotohi Fukuda, and Nobuhiro Ikuno for donating PBC sera.

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