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• CLINICAL RESEARCH •

Construction and expression of a humanized M_2 autoantigen trimer and its application in the diagnosis of primary biliary cirrhosis

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Abstract

AIM: To construct and express a humanized M_2 autoantigen trimer designated as BPO and to apply it in the diagnosis of primary biliary cirrhosis (PBC).

METHODS: cDNA fragments encoding M_2-reactive epitopes of pyruvate dehydrogenase complex E_2 (PDC-E_2), branched chain 2-oxo-acid dehydrogenase complex E_2 (BCOADC-E_2) and 2-oxo-glutarate dehydrogenase complex E_2 (OGDC-E_2) were amplified with PCR using total RNA extracted from human peripheral mononuclear blood cells. The fragments were cloned into the plasmid vector pQE-30 and then transferred into *E. coli* M15 (pREP4) for expression, which was induced by isopropylthio-β-D-galactoside. The expressed recombinant BPO protein was demonstrated by SDS-PAGE, Western-blotting and Immunoabsorption test, its antigenic reactivity and specificity were identified with seven M_2-positive sera confirmed at Euroimmun Research Center (Germany). Using the purified BPO, M_2 antibodies in sera from patients with PBC and other liver related diseases were detected with ELISA.

RESULTS: The expressed BPO was observed with both antigenic reactivity and specificity of M_2 autoantigens. The determination of M_2 antibodies by BPO with ELISA was more sensitive than using the Euroimmun's kit with the coefficients of variation less than 10 % in both interassay and intraassay. With the newly established method, M_2 antibodies were found in 100 % (20/20) of patients with PBC. Six cases of liver disease with unknown etiology and 1 patient with drug induced liver injury had detectable levels of serum M_2 antibodies. There were also 2 patients with autoimmune cholangitis and 1 with autoimmune hepatitis showing M_2-antibody positive.

CONCLUSION: Compared with the routine immunofluorescence assay and commercially available assay kit using porcine heart mitochondrial protein as the antigen, the detection system established in the present study shows higher sensitivity and specificity and may be used as a powerful tool for the diagnosis of PBC.

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronically progressive cholestatic liver disease with autoimmune basis. According to some reports, the incidence of this disease has been consistently increased in recent years[1-4]. One of the remarkable features of PBC is the appearance of high titre antimitochondrial antibodies (AMA) in the patient's sera. Generally, these antibodies are categorized into nine subgroups termed M_1-M_9 according to the antigens they recognize, in which only M_2 antibodies are considered specific for PBC patients that are detectable years or decades before the clinical and histological diagnosis[5-11].

The major autoantigens recognized by M_2 antibodies are the members of 2-oxo-acid dehydrogenase complex including pyruvate dehydrogenase complex E_2 (PDC-E_2), branched chain 2-oxo-acid dehydrogenase complex E_2 (BCOADC-E_2) and 2-oxo-glutarate dehydrogenase complex E_2 (OGDC-E_2), whose immunodominant epitopes have been mapped within lipoyl domains. Antibodies to these corresponding autoantigens have been reported in PBC patients with a positive rate of 95 %, 53-55 % and 39-88 % respectively[6,12]. However, when all of these antibodies are determined simultaneously, the patients with PBC can be diagnosed as high as 92-100 %[13-16]. These facts suggest such a possibility that if there is a constructed antigen containing the specific immunodominant epitopes and the antibodies above can be detected synchronously, the diagnosis of PBC patients would be more specific, sensitive and convenient.

Therefore, we designed and constructed a M_2 autoantigen trimer (BPO) expression vector, which could coexpress the immunodominant lipoyl domains of PDC-E_2, BCOADC-E_2 and OGDC-E_2 from human origin, in an attempt to establish a more accurate and sensitive method with BPO to detect M_2 antibodies in patients with PBC. Besides, because it has never been reported that M_2 antibodies were found in other liver related diseases other than PBC[17-20], a survey to detect M_2 antibodies under these circumstances with our constructed M_2 autoantigen trimer was also included in the present study.

MATERIALS AND METHODS

Patients

Eight groups of adult patients with both sexes who were treated in Shanghai Changzheng Hospital were enrolled in the present study. Group 1 consisted of 20 patients with PBC diagnosed on the criteria: the presence of AMA and at least one of the followings: (1) Elevation of serum alkaline phosphatase (ALP) and/or gamma glutamyl transpeptidase (γ-GT). (2) Liver biopsy with PBC characteristics[21]. Group 2 consisted of 5 patients with autoimmune hepatitis (AIH)[22]. Two patients diagnosed

as autoimmune cholangitis (AIC) were included in group 3, and group 4 was composed of 18 patients diagnosed as liver disease with unknown etiology (LDUE) that was defined as lack of obvious causes including drug use, alcohol abuse, exposure to hepatotoxic medication or chemicals and virus infection. Group 5 consisted of 8 patients with drug induced liver injury (DILI). Group 6 enrolled 201 patients with other liver diseases (Post-viral hepatitis and liver cirrhosis, *n*=153; Obstructive jaundice, *n*=25; Acute hepatitis A, *n*=15; Hepatic abscess, *n*=3; Wilson' s disease, *n*=1; Cardiac cirrhosis, *n*=4). Thirty-three patients with various autoimmune diseases (AID) (Rheumatoid arthritis, *n*=12; Systemic lupus erythmatosus, *n*=12; Polymyositis, *n*=4; Vasculitis, *n*=3; Hashimoto' s thyroiditis, *n*=2) were included in group 7 and 1 225 healthy volunteers taking a health checkup aged less than 28 served as the control. In the experiment, fasting serum from each patient was prepared with routine procedures and stored at -20 ℃ until further analysis.

Materials

Reverse transcriptase and PCR amplification system were purchased from Roche Company (U.S.A). Restriction endonucleases and T_4 DNA ligase were from New England Biolabs (U.S.A). Plasmid vector pQE-30 and *E.coli* M15 (prep 4) were from Qiagen Company (Germany). Indirect immunofluorescence (IIF) test kit for AMA and Western-blotting kit for M_2 antibodies were all from Euroimmun Company (Germany).

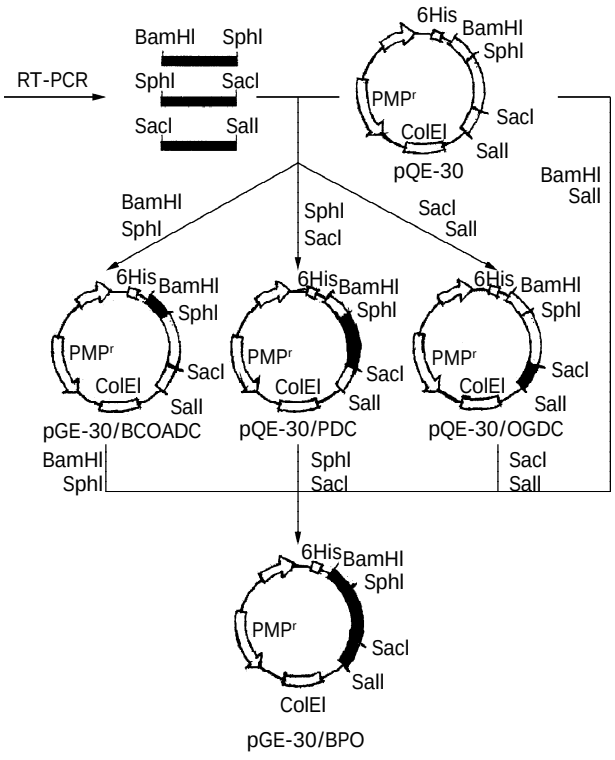

Figure 1 Construction protocol of recombinant plasmids.

Expression and identification of M_2 autoantigen trimer

Recombinant plasmids were constructed as illustrated in Figure 1. Briefly, total RNA was extracted from human peripheral mononuclear blood cells. The objective cDNAs were synthesized by reverse transcriptase and used as the template to amplify the immunodominant epitopes of BCOADC-E_2, PDC-E_2 and OGDC-E_2 with polymerase chain reaction. The PCR products were digested with relevant restriction endonuclease and purified cDNA fragments were inserted into the expression vector pQE-30 to form recombinant plasmids pQE-30/BCOADC-E_2, pQE-30/PDC-E_2, pQE-30/OGDC-E_2 and pQE-30/BPO respectively. The pQE-30/BPO was then transferred into *E. Coli* M15 (pREP4) and induced by isopropylthio-β-D-galactoside to express BPO protein, which was finally confirmed with SDS-PAGE, Western-blotting and Immunoabsorption test.

The antigenic reactivity and specificity of the recombinant BPO trimer were identified with seven M_2-positive sera confirmed at Euroimmun Research Center (Germany) by immunoblotting using beef heart mitochondrial preparations.

Detection of M_2 antibodies with BPO

The obtained recombinant BPO protein fused with the 6×His affinity tag in the N-terminus was purified by Ni-NTA affinity chromatography under denaturing conditions. After renatured by removing denaturants slowly with dialysis, the BPO protein was used as the specific antigen to detect M_2 antibodies with the routine procedures of ELISA. The coefficients of variation for this assay method, the mean OD±SD for the control sera, as well as the critical OD value for the positive determination were respectively calculated or defined based on the experimental results. The measurements of M_2 antibodies and AMA with Euroimmun' s kits as a comparison of the present assay method were also simultaneously performed in the study.

RESULTS

Identification of expressed M_2 autoantigen trimer

The segment analysis by restriction endonuclease digestion confirmed that inserted cDNA sequences in the constructed plasmids were completely consistent with that of the published data (Figure 2). The molecular mass of BPO protein was examined by SDS-PAGE in 15 % polycrylamide gel, in which a specific 42 KD protein band was clearly visualized (Figure 3).

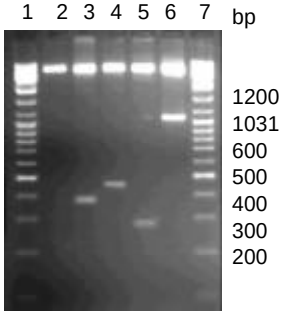

Figure 2 Segment analysis of recombinant plasmids by restriction endonuclease digestion. 1, 7. Markers; 2. pQE-30 (Bamh1); 3. pQE-30/BCOADC-E_2 (Bamh1+Sph1); 4. pQE-30/PDC-E_2 (Sph1+Sac1); 5. p30/OGDC-E_2 (Sac1+Sal1); 6. pQE-30/BPO (Bamh1+Sal1).

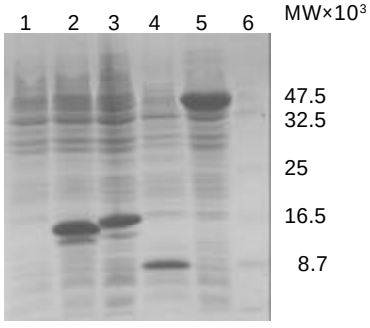

Figure 3 Expression products of recombinant plasmids detected by SDS-PAGE stained with Coomassie Brilliant Blue R-

250. Lane 1: pQE-30 (control); Lane 2: pQE-30/BCOADC-E_2; Lane 3: pQE-30/PDC-E_2; Lane 4: pQE-30/OGDC-E_2; Lane 5: pQE-30/BPO; Lane 6: protein marker.

The expressed BPO protein could react with all of the seven M_2-positive sera confirmed at Euroimmun Research Center (Germany) by immunoblotting using beef heart mitochondrial preparations, which identified the antigenic reactivity of the recombinant BPO trimer (Figure 4). When mixed beforehand with the lysates of *E.coli* expressing BPO overnight, the sera became M_2-negative by Western-blotting, which confirmed the BPO specificity determined by the immunodominant epitopes of PDC-E_2, BCOADC-E_2 and OGDC-E_2.

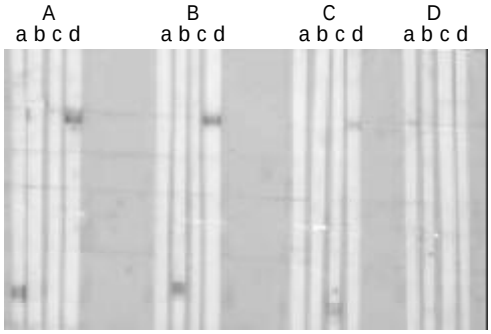

Figure 4 Immunoreactivity of sera against recombinant proteins. Three M_2 antibody positive sera (A, B, C) and a M_2 antibody negative serum (D) were probed with SDS-PAGE-separated recombinant proteins of BCOADC-E_2 (lane a), PDC-E_2 (lane b), OGDC-E_2 (lane c), BPO (lane d).

Effectiveness of BPO in the detection of M_2 antibodies

The coefficients of variation for the detection of M_2 antibodies by BPO with ELISA were less than 10 % in both interassay and intraassay. The mean OD ± SD for the control sera was 0.073±0.046. The critical OD value for positive was defined as ≥ 0.303 based on the mean control value + 5 SD.

M_2 antibodies in patients with PBC

In the patients with PBC who were AMA positive determined by IIF test kit, the positive rate of M_2 antibodies detected by BPO with ELISA and Euroimmun's kit was 100 % (20/20) and 80 % (16/20) respectively (Table 1).

Table 1 AMA and M_2 antibodies in patients with different diseases

Group	*n*	AMA positive	M_2- positive		AASLD's guideline (+)
			Euroimmun's kit	ELISA	
PBC	20	20	16	20	20
AIH	5	0	0	1	0
AIC	2	0	1	2	0
LDUE	18	7	6	6	6
DILI	8	1	1	1	1
Other liver diseases	201	ND	ND	0	ND
AID	33	3	0	0	0
Control	1225	ND	ND	0	ND

PBC: primary biliary cirrhosis; AIH: autoimmune hepatitis; AIC: autoimmune cholangitis; LDUE: liver disease with unknown etiology; DILI: drug induced liver injury; AID: autoimmune disease; AMA: antimitochondrial antibodies; ND: not done.

M_2 antibodies in patients with other diseases

Seven patients with liver disease of unknown etiology were all AMA positive; However, only one was M_2 antibody negative and didn't agree with the guideline by the American Association for the Study of Liver Diseases (AASLD), and his plasma ALP and γ-GT were in normal range but alanine aminotransferase was elevated (120 U/L). The other 6 patients with M_2 antibody positive had no specific symptoms except the unexplained elevation of serum ALP (187-1 525 U/L) and γ-GT (88-2 685 U/L).

One patient with drug induced liver injury was demonstrated as M_2 antibody positive by both ELISA and Euroimmun's kit, whose additional laboratory data were as follows: AMA positive, antinuclear antibodies positive, ALP 153 U/L, γ-GT 321 U/L; alanine aminotransferase 281 U/L, aspartate transaminase 225 U/L, total bilirubin 25 μmol/L (normal < 18 μmol/L). This patient suffered from lymphatic tuberculosis and had taken rifampisin for one year before the onset of liver disease. He was in agreement with the AASLD's guideline.

It was noteworthy that the sera from 1 AIH and 2 AIC patients with AMA negative had detectable M_2 antibody by BPO with ELISA, while they were M_2 antibody negative with the Euroimmun's kit. The prominent elevation of plasma ALP and γ-GT was observed in all of the three patients.

No M_2 antibody positive sera were found in control, other liver disease and the AID group by BPO with ELISA.

DISCUSSION

In the guideline by AASLD in 2000 and the standards by other researchers, AMA has long been used as an important marker for the primary biliary cirrhosis[21, 23]; however, only M_2 antibodies are considered as specific for the PBC diagnosis. Other AMA sub-types have been found in drug-induced disorders, cardiomyopathies, systemic lupus erythmatosus, rheumatoid arthritis, tuberculosis, syphilis and hepatitis C, indicating the nonspecific nature of AMA in the diagnosis of PBC[24]. Besides, there were about 5-17 % of the patients with biochemical and histological features compatible with PBC not having detectable AMA with the IIF method[25-34]. To get better diagnostic results, approaches to detect M_2 antibodies by ELISA or Western-blotting using recombinant antigen of PDC-E_2, BCOADC-E_2 and OGDC-E_2 have been reported in several literatures[15-17].

In 2001, Miyakawa and his coworkers[35] developed a new ELISA for the detection of M_2 antibody using porcine heart mitochondrial protein as the antigen. The sensitivity of this method was only 78 %, despite the specificity was 100 %. In the present study, we employed BPO as the antigen to determine M_2 antibodies with ELISA, which was more sensitive than the Euroimmun's kit. The reason for this was partially because the antigen used in our approach was derived from human sources instead of that from porcine used in Euroimmun's kit. The antigen heterogeneity might affect the assay results[36]. Furthermore, the three major autoantigens, BCOADC-E_2, PDC-E_2 and OGDC-E_2, with no cross-reactivity between, were constructed together as a trimer by molecular biological techniques, which could provide more positive chance for the detection of M2 antibodies. Therefore, the use of this recombinant molecule offered a rapid, simple and sensitive ELISA for the immunodiagnosis of PBC.

According to the investigation by James and his associates[3], the incidence of PBC has been increased in recent years. In northern England, the prevalence of PBC from 201.9 per 10^6 adults and 541.4 per 10^6 women over 40 in 1987 rose to 334.6 and 939.8 respectively in 1994. Owing to the lack of sensitive diagnostic methods, there have no reliable data related to the epidemiology of PBC in China so far and more seriously,

clinical doctors have not yet paid appropriate attention to this disease. We checked 10 patients with liver cirrhosis hospitalized in January, February and April in 2000 whose serum immunological variables showed no signs of viral infection, and the reason for liver cirrhosis seemed unclear. However, 7 of the 10 patients were found M_2 antibody positive by the detailed studies at the Euroimmun Research Center (Germany). In the past six months since we detected M_2 antibody by BPO with ELISA for the PBC diagnosis, over 120 patients' sera have been examined, in which 69 demonstrated M_2 antibody positive and 30 cases with comparatively complete clinical data listed in this paper. Our recent research and the related domestic reports in 2001 indicate that PBC is probably not so rare in China as it has been thought[4, 37].

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