Increased Specificity of ELISA for Anti-mitochondrial Antibody

With a Unique E. coli Buffer: Different Reactivity

Between Primary Biliary Cirrhosis and Collagen Diseases

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Anti-mitochondrial antibody (AMA) is serum hallmark of primary biliary cirrhosis (PBC). However, AMA is also detected in other patients at low frequency. In the present study, 350 patients with various collagen diseases were screened for AMA by enzyme-linked immunosorbent assay (ELISA) using E. coli overexpressing pML-MIT-3 protein as an antigen. Twenty one serum samples showed values higher than the cut-off. Since immunoblotting revealed 14 samples were to be positive for AMA, the AMA-positive rate was 4.0% (14/350). Therefore, seven sera showed non-specific reactions. We hypothesized that the non-specific reactions were due to cross-reactivity with serum non-specific E. coli-derived antibodies and E. coli components of recombinant proteins. So, we added our original E. coli buffer and succeeded in eliminating non-specific reactions. In conclusion, the use of our E. coli buffer can significantly increase the specificity of the ELISA technique for AMA testing, suggesting novel ways to perform routine serum analysis.

Key words: non-specific reaction, unique E. coli buffer, anti-mitochondrial antibody, primary biliary cirrhosis (PBC), collagen disease

Introduction

Primary biliary cirrhosis (PBC) is a chronic progressive cholestatic liver disease characterized by the destruction of small intrahepatic bile ducts and the eventual development of cirrhosis and liver failure9. Presuming an autoimmune etiology, high titers of anti-mitochondrial antibody (AMA) are detected in the sera of most PBC patients9. However, AMA is also detected at low frequency in patients with other diseases9,10, including collagen diseases9,10. AMA has long been measured by immunofluorescence and enzyme-linked immunosorbent assay (ELISA) using rodent-derived proteins as antigens. To increase the sensitivity of the ELISA, different dilutions and binding reagents have been used9.

Recently, major AMA autoantigens were found to belong to the 2-oxo dehydrogenase enzyme complex family: the E2 component of the pyruvate dehydrogenase complex (PDC-E2)9, the E2 component of the branched chain 2-oxo-acid dehydrogenase complex (BCOADC-E2)9, and the E2 component of the 2-oxoglutarate dehydrogenase complex (OGDC-E2)9,10. Using molecular techniques, B-cell epitope mapping has already been done9,10. So, using the three overexpressed recombinant proteins as antigens, novel ELISAs have been developed9,10. In some cases, however, the use of recombinant antigens can also lead to AMA-positivity in non-PBC samples, thus limiting the specificity of the assay.

PBC can be complicated by various collagen diseases such as Sjögren's syndrome and rheumatoid arthritis9. However, the precise relationship be-
Table 1 Subject of various collagen diseases

<table>
<thead>
<tr>
<th>Collagen diseases</th>
<th>Numbers of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>112</td>
</tr>
<tr>
<td>SLE</td>
<td>95</td>
</tr>
<tr>
<td>SSc</td>
<td>27</td>
</tr>
<tr>
<td>MCTD</td>
<td>15</td>
</tr>
<tr>
<td>PM</td>
<td>13</td>
</tr>
<tr>
<td>DM</td>
<td>13</td>
</tr>
<tr>
<td>SJ/S</td>
<td>10</td>
</tr>
<tr>
<td>Vasculitis</td>
<td>17</td>
</tr>
<tr>
<td>Others</td>
<td>48</td>
</tr>
<tr>
<td>Total</td>
<td>350</td>
</tr>
</tbody>
</table>


tween serum AMA and collagen diseases is still unclear.

In the present study, we screened patients with various collagen diseases for AMA by ELISA using a recombinant mitochondrial antigen. Some serum samples gave false-positive results. We hypothesized that such results were due to cross-reactivity with E. coli-derived components in the overexpressed recombinant proteins and serum nonspecific E. coli-derived antibodies. Using a unique E. coli buffer, we succeeded in indicating novel ways to increase the specificity of testing for AMA.

Materials and Methods

Patients

We tested for AMA among 350 patients (mean age: 57 years) with various collagen diseases. All patients had a well-established clinical, immunological, and biochemical diagnosis of collagen disease using established criteria\textsuperscript{13,14}. Diagnoses are listed in Table 1.

Another 20 PBC patients without collagen diseases served as controls. All patients tested positive for AMA by immunofluorescence and ELISA. A diagnosis of PBC was made when two of three criteria (i.e., presence of serum AMA \( \geq 1: 20 \), elevated serum alkaline phosphatase, and compatible liver histology) were met\textsuperscript{15}.

Immunofluorescence

Conventional immunofluorescence for AMA (IF-AMA) was performed using rat liver and kidney frozen sections. A result was considered positive when there was a reaction at a dilution \( \geq 1: 20 \).

ELISA for AMA using a triple expression hybrid clone for three different lipoyl domains (pML-MIT-3-ELISA)

ELISA using the triple expression hybrid clone pML-MIT-3 was performed as described previously\textsuperscript{15}. Briefly, the AMA-reactive immunodominant epitopes within the three distinct lipoyl domains of human PDC-E2, bovine BCODC-E2, and rat OGDC-E2, were cloned and coexpressed in the plasmid vector pGEX-4T-1 (Pharmacia, Alameda, CA) and used as the antigen. Concentration of 4 \( \mu \text{g/ml} \) was dispensed into wells of a microtiter plate (immuno modules microplate, Nunc, France) in volumes of 100 \( \mu \text{l} \) and incubated at 4°C for 24h. After washing three times with PBS containing 0.05% Tween 20, each well was filled with 1% BSA-PBS solution (200 \( \mu \text{l} \)) for postcoating and incubated at 2h, 100 \( \mu \text{l} \) of serum/well (diluted 1: 1,010 with PBS containing 0.15% Tween 20, 1% Casein, and 0.2 M NaCl) were incubated at room temperature for 1 h. After washing three times with PBS containing 0.15% Tween 20, they were incubated with peroxidase-conjugated goat anti-human-IgG, A and M (100 \( \mu \text{l}\) well : MBL, Nagoya, Japan) diluted with freshly prepared buffer (20 mM HEPES, 1% BSA, 0.135 M NaCl, 0.1%, and p-hydroxyphenyl acetic acid) as the secondary antibody. Next, 100 \( \mu \text{l} \) of freshly prepared buffer was added to each well, and the wells were incubated at room temperature for 1 h. After the plates were washed three times with the same buffer, 100 \( \mu \text{l} \)/well of 3,3′,5,5′-tetramethylphenyldiazine was added as substrate, and the plates were incubated at room temperature for 30 min. The enzymatic reaction was stopped by adding 100 \( \mu \text{l} \) of 1.5 N phosphate acid. The optical density (OD) was determined at room temperature using a microplate-reader at 450 nm. The coefficient of variation for both the inter-assay and intra-assay was less than 10%.

Based on our previous data\textsuperscript{15}, the cut-off OD value for determining the AMA was set at mean + 3 S.D. (0.200) of normal control sera. Negative controls were included in all experiments.
The presence of the major 74, 50, and 43-kDa bands was confirmed using sera known to be positive for anti-PDC-E2, anti-BCOADC-E2, and anti-OGDC-E2, respectively. The antigen-specificity of each band was confirmed by inhibition testing as previously described. The 50-kDa and 43-kDa bands were visualized in lane 1 and 2, respectively. The 74-kDa and 50-kDa bands were visualized in lane 3. N.C: negative control. PC: positive control.

**Preparation of E. coli buffer**

*E. coli* DH5 alpha was used to overexpress the recombinant protein as antigen in pML-MIT-3-ELISA and bacterial lysates were prepared as previously described. Briefly, plasmid DNA (PGEX-4T) was added to *E. coli* DH5 alpha competent cells, which were dissolved on ice and mixed. After heat shock treatment (42°C for 30 sec), sterile SOC medium was added to the culture followed by shaking, and cells were then seeded on LB agar plates. After transformation, the cultured *E. coli* was rinsed, then frozen and thawed several times, sonicated eight times for 30 sec and centrifuged at 15,000 rpm to obtain the supernatant. The final protein concentration in the supernatant was 32.1 mg/dl as measured by the Bradford Protein Assay (Bio-Rad, Hercules, CA).

**Use of a unique E. coli buffer in ELISA for AMA**

All serum samples with an OD value higher than the cut-off were further tested for AMA with ELISA using the unique buffer. Sera were tested against antigens obtained with and without the unique buffer and OD values were calculated.

**Immunoblot analysis for AMA**

An immunoblot analysis for AMA, using a bovine heart mitochondrial fraction, was performed as described previously. After SDS-PAGE was performed, electroblotting onto a polyvinylidene difluoride (PVDF; Millipore, Bedford, MA) membrane was conducted using a disk-type blotting apparatus (Nippon Eido, Tokyo, Japan). Patients’ sera were used as the first antibody at a 1: 100 dilution. Peroxidase-conjugated goat anti-human IgG, IgA and IgM (MBL, Nagoya, Japan) at an optimal final dilution of 1: 3,000 was used as the secondary antibody. The PVDF strips were developed with 3,3'-diaminobenzidine (0.1 mg/ml) in 50 ml of PBS [pH 7.4], and 0.06% H2O2. The presence of the major 74, 50, and 43 kDa bands was confirmed using known positive sera for anti-PDC-E2, anti-BCOADC-E2, and anti-OGDC-E2, respectively. Fig. 1 illustrates the immunoblot analysis for representative sera. The antigen specificity of each band was confirmed by inhibition tests using each recombinant protein as previously described.

**Statistical analysis**

Group data were expressed as the mean ± SD and compared with the Welch’s t-test, paired T, and the chi-square test. A p value <0.05 was considered statistically significant and all analyses were twotailed.

**Results**

**AMA-based screening by ELISA**

Among 350 serum samples, 21 had an OD value higher than the cut-off with ELISA. All 10 samples positive for AMA by immunofluorescence were included.

**Immunoblot analysis for AMA**

We further analyzed for AMA in the 21 samples by immunoblotting using the bovine heart mitochondrial fraction as antigen. Fourteen samples were positive for at least one of anti-PDC-E2, anti-BCOADC-E2, and anti-OGDC-E2. The other seven were all negative for AMA.

Based on the immunoblot analysis, the AMA-positive rate in various collagen diseases was 4.0% (14/350). Thus, in the present study, the remaining seven sera gave false-positive results for AMA. We divided the 21 sera into two groups; an AMA-positive group (Group A, n=14) and an AMA-negative group (Group B, n=7) based on the immunoblot analysis. All results are summarized in Table 2.
Table 2  AMA analyzed by IF, ELISA, and immunoblotting in 21 sera from patients with various collagen diseases

<table>
<thead>
<tr>
<th>No</th>
<th>Patient</th>
<th>Age/Sex</th>
<th>Diagnosis</th>
<th>AMA (ELISA)</th>
<th>AMA (IF)</th>
<th>IB (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>74</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>S.M.</td>
<td>55/F</td>
<td>RA</td>
<td>1.060 (+)</td>
<td>1:320</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>K.M.</td>
<td>56/F</td>
<td>DM</td>
<td>1.399 (+)</td>
<td>1:160</td>
<td>G</td>
</tr>
<tr>
<td>3</td>
<td>F.S.</td>
<td>59/M</td>
<td>Vasculitis</td>
<td>3.000 (+)</td>
<td>1:80</td>
<td>GAM</td>
</tr>
<tr>
<td>4</td>
<td>O.A.</td>
<td>78/F</td>
<td>RA</td>
<td>1.511 (+)</td>
<td>1:80</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>S.K.</td>
<td>59/F</td>
<td>SSc</td>
<td>1.239 (+)</td>
<td>1:80</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>I.Y.</td>
<td>64/F</td>
<td>SLE</td>
<td>0.812 (+)</td>
<td>1:80</td>
<td>G</td>
</tr>
<tr>
<td>7</td>
<td>T.Y.</td>
<td>64/F</td>
<td>SSc</td>
<td>0.873 (+)</td>
<td>1:40</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Y.N.</td>
<td>60/M</td>
<td>SI/S</td>
<td>0.663 (+)</td>
<td>1:40</td>
<td>G</td>
</tr>
<tr>
<td>9</td>
<td>K.Y.</td>
<td>80/F</td>
<td>Vasculitis</td>
<td>0.471 (+)</td>
<td>1:20</td>
<td>G</td>
</tr>
<tr>
<td>10</td>
<td>Y.F.</td>
<td>56/M</td>
<td>SSc</td>
<td>0.213 (+)</td>
<td>1:20</td>
<td>-</td>
</tr>
<tr>
<td>11</td>
<td>K.K.</td>
<td>55/F</td>
<td>SSc</td>
<td>0.598 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td>T.Y.</td>
<td>49/F</td>
<td>PM</td>
<td>0.537 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>M.Y.</td>
<td>23/F</td>
<td>SLE</td>
<td>0.498 (+)</td>
<td>negative</td>
<td>GA</td>
</tr>
<tr>
<td>14</td>
<td>N.Y.</td>
<td>38/F</td>
<td>SLE</td>
<td>0.376 (+)</td>
<td>negative</td>
<td>G</td>
</tr>
<tr>
<td>15</td>
<td>N.M.</td>
<td>36/F</td>
<td>MCTD</td>
<td>0.453 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>16</td>
<td>S.M.</td>
<td>30/F</td>
<td>SLE</td>
<td>0.427 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>17</td>
<td>W.K.</td>
<td>76/F</td>
<td>RA</td>
<td>0.410 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>18</td>
<td>R.Y.</td>
<td>67/F</td>
<td>RA</td>
<td>0.343 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>19</td>
<td>Y.K.</td>
<td>47/M</td>
<td>Vasculitis</td>
<td>0.329 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>F.Y.</td>
<td>23/F</td>
<td>SLE</td>
<td>0.317 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
<tr>
<td>21</td>
<td>F.E.</td>
<td>19/F</td>
<td>SLE</td>
<td>0.286 (+)</td>
<td>negative</td>
<td>-</td>
</tr>
</tbody>
</table>


There were no significant differences in mean age (56.9±14.3 vs 42.5±21.0, p=ns), male to female ratio (3/11 vs 1/6, p=ns), and collagen disease category between the groups. All 10 IF-AMA-positive patients were included in group A. Immunoblotting in group A revealed six, nine, and two sera to be positive for anti-PDC-E2 (74-kDa), anti-BCOADC-E2 (50-kDa), and anti-OGDC-E2 (43-kDa), respectively.

**Efficacy of a unique E. coli buffer for recombinant AMA-ELISA**

To investigate the effects of our unique E. coli buffer on the ELISA for AMA, we compared OD values obtained with and without the buffer. Fig. 2 illustrates the changes in OD values among the three conditions. PBC sera (n=20) produced similar OD values when the buffer was added (2.193 ± 0.880 vs 2.299 ± 0.859 without the buffer, p=ns). When the 14 sera from group A were used, similar mean OD values produced when the buffer was added (0.893 ± 0.714 vs 0.946 ± 0.708 without the buffer, p=ns), however, interestingly, when the seven sera from group B were used, the addition of our E. coli buffer led to significantly lower OD values (0.176 ± 0.018 vs 0.366 ± 0.063 without the buffer; p<0.01). When our E. coli buffer was used, the decrease in OD values led to AMA-negativity in all cases in group B, by contrast, all PBC sera and 13 of 14 sera of group A still had values higher than the cut-off OD value with the buffer.

**Discussion**

PBC is diagnosed based on the presence of AMA in serum, biochemical data, and liver histological findings such as non-suppurative destructive cholangitis and bile duct loss. However, liver biopsy specimens do not always provide such findings, especially in the disease’s early stages. Therefore, the presence of AMA is important for the diagnosis of PBC. In the present study, we screened patients with various collagen diseases for AMA by ELISA using recombinant mitochondrial proteins as antigens. ELISA is useful for screening AMA, as well as other antibodies, because of its accuracy and ease of performance, however, since hyper-gammaglobulinemia and several autoantibodies are observed in patients with collagen diseases, a strict judgment regarding the presence or absence of the
autoantibody is needed. Among the 350 serum samples tested, 21 showed OD values higher than cut-off value. Based on immunoblot analyses, AMA-positive rate among the patients with various collagen diseases was 4.0% (14/350). This result is almost consistent with the study by Matsumoto et al., however, the rate is significantly higher than the AMA-positive rate among Japanese corporate workers (0.64%)17. The disease deflection was not observed in AMA-positive patients. Interestingly, based on biochemical data including serum alkaline phosphatase levels, in our AMA-positive 14 patients, 11 patients were clinically associated with PBC. The remaining three patients who categorized in so-called early PBC were needed a careful follow-up study. James et al. described increasing disease spectrum of PBC cases with various collagen diseases10.

We believe that the most sensitive method of detecting AMA is immunoblotting. However, the technique is too complicated for routine use. Therefore, we need to increase the specificity of ELISAs.

We hypothesized that the false-positive results were due to cross-reactivity with serum non-specific E. coli-derived antibodies and E. coli components of recombinant proteins. When the E. coli buffer was added, as shown in Fig. 2, group A sera had not significantly reduced OD values, similar to PBC sera, however, group B sera had significantly reduced OD values. After the buffer was added, all group A patients except one tested positive with the ELISA. It is of note that the results obtained using the E. coli buffer are almost concordant with those of the immunoblot analysis of AMA. Therefore, we suggest that the sensitivity of the ELISA for AMA is increased by the buffer.

Bacterial infections may contribute to B cell immunity via the activation of a polyclonal antibody production and possibly the release of isolated antigens that could in turn activate the innate immune cascade. Bacterial molecules such as lipopolysaccharides and CpG might then act as modulators of the immune response by binding to toll-like receptors on macrophages and dendritic cells19.

We demonstrated that reactivity to recombinant mitochondrial antigens, albeit at low titers, can be observed also in sera from some patients with collagen diseases. We previously compared anti-PDC-E2
reactivity between PBC and non-PBC sera by conducting dilution tests\textsuperscript{20}. Results showed that at a dilution of 1: 100, 100% of anti-PDC-E2-positive PBC and up to 38% of control sera recognized proteins corresponding to E.coli PDC-E2, while at a dilution of 1: 10,000, still 92% of PBC and only 6% of non-PBC control sera recognized these proteins. These dilution tests showed that the overall potency of PBC reactivity was increased 100-fold compared to the controls. This result suggests that the AMA in PBC is a high-affinity antibody binding to bacterial proteins, and not be affected by the addition of E.coli buffer.

The current study of the collagen diseases indicates that the frequency of non-specific reactions can be reduced by using our unique E.coli buffer. The devise can be applied to other ELISA systems using recombinant antigen.

In conclusion, the use of the E. coli buffer can significantly increase the specificity of the ELISA technique for AMA testing, thus suggesting novel ways to perform routine serum analysis.

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References

新規の大腸菌 buffer 使用による、ELISA 法での AMA の特異性の上昇—PBC とその他の膵原病患者における反応性の相違

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抗ミトコンドリア抗体 (AMA) は原発性胆汁性肝硬変 (PBC) における特異的な血清学的診断マーカーである。しかしながら、PBC 以外の疾患でも低頻度ながら検出される。本研究では、細菌内で大量生産された pML-MIT3 蛋白を抗原とした ELISA によって各種膵原病 350 例の AMA をスクリーニングした。21 例がカットオフ値を上回った。免疫プロット法による解析では 14 例に AMA が検出されたので、最終的に膵原病における AMA 阳性率は 4.0% であった。すなわち、他の 7 例は非特異反応であった。この非特異反応は血清中の非特異的な E. coli 由来の抗体と、組み換え蛋白の E. coli 組成との交差反応によって惹起されるとの仮説を立てた。そこで、独自の unique E. coli buffer をこの ELISA 系に加えることによって、膵原病における非特異反応を消失させることができた。結論として、この E. coli buffer の使用は AMA 測定系の特異性を有意に向上させることから、ルーチンの血清学的分析のための新しい方法となり得ることが示唆された。