Simultaneous Analysis of Microheterogeneity of Immunoglobulins and Serum Protein Fraction Using High-Voltage Isoelectric Focusing on Six Cellulose Acetate Membranes

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A systematic detection method for the single performance of cellulose acetate (CA) membrane isoelectric focusing to detect six different types of information on protein abnormalities was developed. High-voltage isoelectric focusing was carried out on six layers of CA membrane using a thermoelectric cooling apparatus. After electrophoresis, the proteins on the top, the third, the fourth, the fifth, and the bottom CA membrane were transferred to a polyvinylidene difluoride (PVDF) membrane by a simple contact printing procedure to detect IgM, κ -chain, λ -chain, IgA, and IgG, re-

spectively. Each PVDF membrane revealed the microheterogeneity of these immunoglobulins using specified anti-serum and enzyme immunostaining. The second CA membrane was stained with Coomassie brilliant blue G250 to detect serum protein patterns. All stained membranes showed clear electrophoretic patterns of immunoglobulin microheterogeneity. By our method, immunoglobulin abnormalities in serum could be screened out using six different types of information obtained simultaneously. *J. Clin. Lab. Anal.* 11:220–224, 1997. © 1997 Wiley-Liss, Inc.

Key words: cellulose acetate membrane; isoelectric focusing; microheterogeneity of human serum immunoglobulin; human serum protein

INTRODUCTION

We have developed an apparatus for isoelectric focusing using cellulose acetate (CA) membranes, and optimized the procedures (1,2). Isoelectric focusing has been shown to be a useful technique for protein analysis. This technique has especially been used for the detection of immunoglobulin abnormalities in various diseases. For example, oligoclonal immunoglobulins have been detected and identified in the serum of patients with human immunodeficiency virus with infection (3), chronic viral hepatitis (4), multiple sclerosis (5), and in the serum of an organ transplant recipient (6).

Monoclonal immunoglobulins appear in the serum of patients with multiple myeloma, primary macroglobulinemia, and essential M proteinemia (7). Immunoglobulin microheterogeneity by the isoelectric focusing method may be a useful tool to obtain detailed disease information. Herein, we developed a systematic detection method for the single performance of CA membrane isoelectric focusing to detect six different types of information on the immunoglobulin microheterogeneity of sera: IgM, IgA, IgG, κ -chain, λ -chain, and protein patterns.

MATERIALS AND METHODS

Chemicals

Separax EF (CA membrane) and 20% Sepaline 3.5-10 (carrier-ampholyte) were from Fuji Photo Co. (Japan). Forty percent SERVALYT 4-6, 40% SERVALYT 9-11 (carrier-ampholyte) were from Serva Feinbiochemica GmbH & Co. (Germany). Coomassie brilliant blue G-250 was from Sigma Chemical Co. (USA). Rabbit anti-human IgG immunoglobulin (lot no. 128), rabbit anti-human IgM immunoglobulin (lot no. 128), rabbit anti-human IgA immunoglobulin (lot no. 018), rabbit anti-human κ -chain immunoglobulin (lot no. 100C), rabbit anti-human λ -chain immunoglobulins (lot no. 051B), and peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (lot no. 119) were from Dako Chemicals (Sweden). Blockace (blocking solution) was from

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Dainihon Pharmaceutical Co. (Japan). Dulbecco's PBS(−) buffer was from Nissui Pharmaceutical Co. Ltd. (Japan). Other chemicals of reagent grade were purchased from Wako Pure Chemicals (Japan).

Samples

Serum samples were collected from seven inpatients at the Tokyo Medical and Dental University Hospital. After we confirmed the presence of M protein in these sera using CA membrane electrophoresis, we determined the type of M protein using immunofixation. The M proteins were IgG- κ , IgG- λ , IgG- λ -IgM- λ , and IgA- κ in 3, 2, 1, and 1 serum samples, respectively. Serum from a normal volunteer was used as control.

Isoelectric Focusing on Multilayer Membrane

We used a thermo-module cooling isoelectric focusing apparatus (HIPEP ISOPHOR MK-1, COSMO BIO, Japan). The conditions for electrophoresis were determined according to our method (2). The carrier-ampholyte solution was prepared from mixed 20% Sepaline 3.5-10, 20% Sepaline 4-6, 40% SERVLYT 9-11, and 20% saccharose in a mixing ratio of 10 : 10 : 1 : 21. The CA membranes were soaked with carrier-ampholyte solution for 5 minutes. One by one, six sheets of CA membrane were overlaid on a ceramic cooling bed. The temperature of the CA membranes was kept at 7°C during the performance of isoelectric focusing. The 30-minute preliminary electrophoresis at 500 V was followed by sample application (1 μ l) and stepwise electrophoresis as follows: at 300 V for 10 minutes, 500 V for 30 minutes, 1,500 V for 45 minutes, and 2,000 V for 5 minutes.

Protein Staining

After electrophoresis, the six sheets of CA membrane were peeled off one by one. The second CA membrane was stained with Coomassie brilliant blue G250 according to the method of Toda et al. (1).

Immunoblotting

Five sheets of polyvinylidene difluoride (PVDF) membrane (Immobilon-PSQ, Millipore, USA) and filter paper (70 $\times$ 120 mm) were wetted with transfer buffer (0.025 M Tris-0.192 M glycine-4% methanol-0.05% sodium dodecyl sulfate). Each of the five CA membranes, except the second, was overlaid with wetted PVDF membrane and wetted filter paper. These membranes were allowed to stand overnight in a moisture box for blotting. After blotting, the PVDF membranes were blocked for 1 hour with a blocking solution. These PVDF membranes were washed in T-PBS (containing 0.05% Tween 20 in phosphate buffer saline) for 3 $\times$ 1 minute. Anti-human IgG and IgA antibodies were diluted 1,000 times using T-PBS. Anti-human IgM, κ -chain, and λ -chain antibodies were

diluted 2,000 times using T-PBS. The PVDF membrane to which the top CA membrane had been transferred was immersed in diluted anti-IgM solution, the PVDF membrane to which the third CA membrane had been transferred was immersed in diluted anti- κ -chain solution, the PVDF membrane to which the fourth CA membrane had been transferred was immersed in diluted anti- λ -chain solution, the PVDF membrane to which the fifth CA membrane had been transferred was immersed in diluted anti-IgA solution, and the PVDF membrane to which the bottom CA membrane had been transferred was immersed in diluted anti-IgG solution. Each PVDF membrane was incubated for 1 hour at room temperature and washed in T-PBS for 3 $\times$ 5 minutes. Peroxidase-conjugated goat anti-rabbit immunoglobulin antibody was diluted 2,000 times using T-PBS. Then, all the PVDF membranes were immersed in diluted peroxidase-conjugated anti-rabbit immunoglobulin goat antibody solution for 1 hour. After incubation, these PVDF membranes were washed in T-PBS for 3 $\times$ 5 minutes. The development solution consisting of 0.006% H₂O₂ and 0.02% diaminobenzidine using T-PBS was prepared before use. The PVDF membranes were immersed in the development solution for 5 minutes.

Analysis of Electrophoretic Pattern

Image analysis of electrophoretic patterns was performed on a Macintosh 6100/60AV computer using the public domain NIH Image program.

Measurement of pH Gradient

Electrophoresis was performed by the same method except for sample application. After electrophoresis, all six CA membranes were cut into 5 mm-wide strips from the cathode side. Twenty-two pieces of strips were divided and each strip was put into a glass tube in 1 ml of distilled water. The carrier ampholyte in each piece was eluted into distilled water with continuous shaking for 1 hour. The pH of each solution was determined by pH meter (Horiba, Japan).

RESULTS

After electrophoresis, it is possible to peel the sheets of CA membrane one by one. On the basis of this characteristic, we tried various procedures. First, we confirmed how protein patterns are formed in each CA membrane. After electrophoresis, all six CA membranes were stained with Coomassie brilliant blue G250. Figure 1 shows the isoelectric patterns for serum proteins from seven patients with M protein (lanes 1–7) and a healthy subject (lane 8). All of the CA membranes showed clear electrophoretic patterns. However, the number of detection bands decreased in the lower layers of CA membrane. In the top

Fig. 1. Electrophoretic patterns of human serum proteins obtained by isoelectric focusing on six layers of CA membrane. Six membranes were stained with Coomassie brilliant blue G250. **Lanes 1–7:** M protein serum; **lane 8:**

a healthy subject, Column. The top (1), second (2), third (3), fourth (4), fifth (5), and bottom membranes (6) were stained with Coomassie brilliant blue G250 method.

and second CA membranes, many serum protein bands were detected. In the third and fourth CA membranes, albumin, M protein, and some serum protein bands were detected. In the fifth and bottom CA membranes, hardly any proteins were detected, except albumin. To determine the reproducibility of protein distribution, we examined the distribution of protein among the six layers of CA membrane using control serum (Fig. 2). The optical density of protein in the top CA membrane was the highest of the six CA membranes. The lower the CA membrane was

the lower the protein concentration. The protein distribution of each CA membrane showed good reproducibility. Figure 3 shows the pH gradient curve. The coefficient of correlation of pH gradient was 0.986.

Simultaneous Detection of IgM, IgA, IgG, κ -Chain, and λ -Chain Microheterogeneity

The present method provides six different types of information simultaneously. Thus, we attempted the simultaneous detection of serum IgM, IgA, IgG, κ -chain,

Fig. 2. Distribution of serum protein among six layers of CA membrane. After electrophoresis, all CA membranes were stained with Coomassie brilliant blue G250. The optical density of distribution of control serum (Fig. 1, lane 1) was measured by an NIH image program. Each value represents the mean $\pm$ SD of four experiments.

λ -chain microheterogeneity, and protein fraction using six CA membranes. We performed a preliminary experiment to determine the suitable conditions: the top CA membrane was suitable for IgM detection, the second for pro-

tein fraction detection, the third for κ -chain detection, the fourth for λ -chain detection, the fifth for IgA detection, and the bottom CA membrane for IgG detection. All sheets showed clear electrophoretic patterns of immunoglobulin microheterogeneity (Fig. 4). Lanes 2, 4, and 6 were identified as IgG κ -chain type using immunofixation. However, although they were of the same type, the pI of the main IgG band was different by our method. Moreover, not one band but rather two or three bands close to the main band were strongly stained.

DISCUSSION

The most important characteristic of this method is that isoelectric focusing can be performed by overlaying CA membranes. With this method, it is also possible to peel off the CA membranes one by one after electrophoresis and use a different detection method for each CA membrane. This is not possible when using polyacrylamide and agarose gels generally employed for isoelectric focusing. In this study, we used six layers of CA membrane for the single performance of electrophoresis. Protein distribution among six layers of CA membrane has been discussed by Toda et al. (8) and it was shown that high molecular weight proteins were not likely to permeate evenly among the layers compared to small molecules. The fact that the degree of protein permeation in the six CA membranes differs according to protein molecular weight does not affect data recognition because the analysis of the same protein is determined by the same layer of CA membrane and protein distribution on each membrane thus remains constant. We were able to determine the concentration of each immunoglobulin with microheterogeneity because of the decrease in protein amount from the top to the bottom membrane. In this study, we were able to avoid using polyacrylamide and agarose gels which are not easy to handle and cannot be overlaid. As reported previously, when three CA membranes are overlaid for electrophoresis, results are consistent from band to band (2). We obtained the same results using six CA membranes. In the present method, the mobility distance remained constant because a single electrophoresis was performed on six overlaid CA membranes. With this method, it is possible to detect proteins with very close isoelectric points. This method could be a useful tool for the analysis of the clinical condition of immunoglobulin abnormalities. The additional advantage of this method is that it allows for six sets of information to be obtained simultaneously because six different staining techniques are used at once.

Fig. 3. Determination of pH gradient profile using a CA membrane.

Fig. 4. Six different aspects of serum protein abnormalities detected simultaneously by a single performance of isoelectric focusing on six layers of CA membrane. For the top membrane (1), IgM detection was used; for the second membrane (2), serum protein detection; for the third membrane (3), κ -chain detection; for the fourth membrane (4), λ -chain detection; for

fifth membrane (5), IgA detection; and for the bottom membrane (6), IgG detection. **Lane 1:** IgG- λ -IgM- λ type M protein serum; **lanes 2, 4, 6:** IgG- κ type M protein sera; **lane 3:** IgA- κ type M protein serum; **lanes 5, 7:** IgG- λ type M protein sera; **lane 8:** control serum.

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