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Native flat-bed isoelectric focusing for determining antibody clonotype distribution

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Abstract

A sensitive method to analyze specific antibody clonotype changes in a patient with systemic lupus erythematosus, who developed autoantibodies to the Ro 60 autoantigen under observation, is described in this chapter. Patient sera collected over several years were separated by flatbed isoelectric focusing (IEF). Following electrofocusing, the gel is now ready to be analyzed by affinity immunoblotting utilizing Ro 60-coated nitrocellulose membrane to determine oligoclonality of the anti-Ro60 containing sera.

Keywords

Affinity immunoblotting; Clonotype distribution; Systemic lupus erythematosus; Ro 60 autoantigen; Flatbed IEF

1. Introduction

Isoelectric focusing (IEF) serves as a useful procedure for studying the heterogeneity of antibody and immunoglobulin (Ig) clonotypes (1). Patterns of antigen-specific antibody clonotype can tell whether changes in cell population happen during ongoing immune responses as a response to regulatory influences. It can also show whether changes in hybridoma cell lines can occur with time (2). Previous studies used to investigate these changes by immobilizing the separated antibody clonotypes following IEF and incubating them with radioactive antigen. Radiolabeled hapten was allowed to diffuse into a gel, in one method, prior to precipitation of Ig with sodium sulfate followed by detection of hapten-specific clonotype distribution by autoradiography (3). Ig was precipitated in the gel with sodium sulfate, in another study, immediately following completion of the focusing run. It was then crosslinked with glutaraldehyde followed by the addition of labeled antigen or anti-Ig (4). Subsequent studies showed that fixation with glutaraldehyde could diminish the antigen-binding capability of certain Ig (5). In addition, it was shown that the earlier study

was unable to define optimal crosslinker (glutaraldehyde or suberimidate) concentration, since certain antibodies could not be fixed at crosslinker concentrations that substantially inactivated others. The excess time needed to diffuse antigen into the gel and for rinsing the unbound antigen out of the gel is another drawback of these methods, which can take several days especially when using radioactive probes.

One procedure for immobilizing focused antibodies entailed the use of nitrocellulose membranes. Focused antibodies are transferred electrophoretically or non-electrophoretically to nitrocellulose membrane and labeled antigen was employed to detect antigen specific clonotypes (6). Yet another procedure involved laying the gel with the focused antibodies with agarose containing antigen-coated sheep erythrocytes (7). In this procedure, antibodies diffuse into the RBC-containing gel, bind the antigen-coated cells and lyse the cells after complement addition.

In this chapter, we describe a procedure in which a 60,000 molecular weight Ro autoantigen was first passively immobilized on nitrocellulose membrane and placed in contact with an IEF gel containing autoantibodies (derived from a patient with systemic lupus erythematosus who developed antibodies to the Ro 60 autoantigen over time) focused according to its isoelectric point. After diffusion mediated transfer to membrane the non-specific antibody clonotypes are removed by washing and the antigen-specific antibody clonotypes are detected using alkaline phosphatase conjugated anti-Ig (8).

Systemic lupus erythematosus (SLE) is a complex, chronic autoimmune disease in which autoantibodies target self-antigens, including the Ro (or SS-A) ribonucleoprotein complex. Anti-Ro 60 antibodies occur in up to 40% of patients with SLE (8). The epitopes of the Ro 60 autoantigen targeted by SLE patients have been characterized previously (9,10). Even though anti-Ro 60 sera commonly bound short Ro 60 peptides, it did not bind the denatured Ro 60 antigen well. In addition, the antibodies that bound Ro 60 octapeptides were also found to bind the native protein (10).

Examples of some SLE autoantibodies appearing and disappearing, at times in association with specific disease manifestations, therapy or generalized clinical disease activity have been reported. For example, antibodies to native DNA is associated with renal disease, and the detection of this autoantibody may be an indication of disease exacerbation (11). Antibodies to the P autoantigen (ribosomal P antigens) can appear with an increase of neurologic or renal disease. Autoantibodies like anti-Ro 60, on the other hand, occur in some normal subjects as well as in SLE patients prior to onset of disease (8), and develop only rarely during the course of SLE.

This study was carried out after the identification of a patient with SLE who developed antibodies to the Ro 60 autoantigen after about 10 years of illness. Figure 3 shows that anti-Ro 60 clonality increases in complexity, and affinity to the Ro 60 antigen also increases as the response developed.

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω cm at 25°C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing waste materials. We do not add sodium azide to the reagents.

1. 25% glycerol (v/v): Add 25 mL glycerol to 75 mL of distilled water. Mix well.
2. 5x acrylamide (26.5% T, 3% C): Add about 25 mL water to a 100 mL graduated cylinder or a glass beaker. Weigh 12.84 g acrylamide and 0.4098 g bis acrylamide and transfer to the cylinder (*see* Note 1). Add a spatula of AG 501-X8 (D) mixed-resin beads (Bio-Rad, Hercules, CA, USA) and stir using a magnetic stir bar on a magnetic plate for about 30 minutes. Make up to 50 ml (after removing the stir bar) with water and filter through a 0.45 μ m Corning filter (*see* Note 2). Store at 4°C, with bottle wrapped with aluminum foil (*see* Note 3).
3. 10% Tween-20: Add 90 mL of distilled water into a glass beaker. Add 10 mL Tween-20 and mix.
4. 2% ammonium persulfate: Weigh 0.02 g ammonium persulfate and dissolve in 1 mL of distilled water (*see* Note 4).
5. N.N.N. -Tetramethyl-ethylenediamine. Store at 4°C (*see* Note 5).
6. Alkaline phosphatase buffer: Weigh 6.1 g of Tris, 2.9 g sodium chloride and 0.51 g magnesium chloride-6H₂O and make it to 500 mL with water after adjusting pH to 9.3 with HCl (*see* Note 1). Store at 4°C.
7. Nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP): Dissolve 1 g NBT in 20 mL of 70% dimethylformamide (DMF). Dissolve 1 g BCIP in 20 mL of 100% DMF. Add 33 μ L of BCIP and 66 μ L of NBT to 10 mL of alkaline phosphatase buffer just before adding to membrane.
8. Nitrocellulose membrane.
9. 0.5 M sodium bicarbonate solution, pH 9.5.
10. Phosphate buffered saline (PBS), pH 7.4.
11. PBS containing 0.05% Tween-20 (PBST).
12. Ampholytes: pH 3–10 and pH 8–10.5 (Amersham/GE Healthcare, Piscataway, NJ, USA).
13. Ro 60 autoantigen (Immunovision, Springdale, AK, USA).
14. Glass plates: Two 5" by 4" glass plates.
15. Medium binder clips (1 1/4 inch).
16. Small binder clips (3/4 inch).

17. Gasket with 3 edges, about 3 mm wide, to serve as spacer between the plates.
18. LKB-2117 Multiphor apparatus for IEF (LKB Instruments, Rockville, MD, USA).
19. Model 3000/300 power supply (Bio-Rad).
20. pH 3 and pH 10 solutions (Serva Electrophoresis GmbH, Heidelberg Germany).
21. Helium gas.
22. Sample applicator strip (Bio-Rad).
23. Paper wicks.

3. Methods

All procedures are at room temperature unless otherwise specified.

1. Pipet 5.6 mL of distilled water into a conical flask. Add 2 mL of 25% glycerol followed by 2.1 mL of the 5x acrylamide solution. Then add 300 μ L of pH 3–10 ampholytes followed by 100 μ L of pH 8–10.5 ampholytes.
2. Degas this solution by bubbling helium through it for 15 min. Rinse the metal end of degassing tube first with water and wipe dry with Kimwipes.
3. While the solution is degassing, set up the gel apparatus. Soak the gasket in water for few min. Mop dry with Kimwipes.
4. Take one glass plate and lay the gasket on top of the glass plate around the edges so that it will seal the bottom and two sides of the plates. Lay the other glass plate on top of the gasket. Clamp the clips around the edges of the plates (bottom, the left side and the right side (see Fig. 1). Stand the gel upright on using the base of the clips (see Fig. 1) to pour the gel. Prepare a 2% ammonium persulfate solution fresh.
5. After degassing is complete, the metal end of the degassing tube is cleaned with water. To the degassed solution, add 100 μ L of 10% Tween-20 and mix gently. Then add 100 μ L of 2% APS. Have a Pasteur pipette ready for pouring the gel. Add 10 μ L of TEMED and mix gently. Pipet the gel mixture into the Pasteur pipette and transfer into the gel apparatus quickly. Attempts should be made to avoid bubbles. Fill up the gel apparatus to the top. Polymerization should begin within minutes. However, let the assembly stand for two h without disturbance.
6. Turn cooling unit on and set it on 4°C in preparation for focusing.
7. After two h carefully remove one of the glass plates and gasket. The gel will remain on one of the glass plates.
8. Lay the glass plate on top of the IEF unit, with the gel side facing up (wipe of water on top of the unit beforehand). Place the smaller cover in place and press down slightly so as to make imprints for the wicks. Cut two wicks to the size of the gel (be as close as possible). Soak the top wick in Serva pH 3 solution and

the bottom wick in Serva pH 10 solution. Dab off excess solution and place where imprints were made by cover (see Fig. 2).

9. Put smaller cover back on, making sure connection is made with both wicks. Connect red and black wires. Put on larger cover and make connections to power supply (red = +^{ve}; black = -^{ve}).
10. Prefocus by setting constant voltage 200 V for 20 min, then increase voltage to 400 V for another 20 min. Prepare samples for application.
11. Turn off power supply, disconnect wires, and remove covers. Take applicator strip and lay on top of gel 1–2 cm below top wick. Make sure strip is stuck to the gel well. Strip can hang over gel a little (see Fig. 2; see Note 6).
12. Apply the samples, being careful not to spill over into other wells. Replace the covers and make the connections. Turn the power supply on to 12 W constant power. Focus for approximately 1–2 h. When focusing, the voltage will rise, and the current will drop. The rate at which these two parameters change is much faster in the beginning than the end. The run is complete when the voltage is between 1800–2000V and the current 3–5 A. When the change appears to be very slow or not at all, turn off the unit (see Note 7).
13. After the run is complete, transfer the focused protein from the gel to membrane. Take gel off the flatbed and remove applicator strip.
14. Stain gel with appropriate stain or carry out transfer to membrane (12,13).

4. Notes

1. Having water at the bottom of the cylinder helps to dissolve the Tris relatively easily, allowing the magnetic stir bar to go to work immediately. If using a glass beaker, the Tris can be dissolved faster if the water is warmed to about 37°C. However, the downside is that care should be taken to bring the solution to room temperature before adjusting pH.
2. Wear a mask when weighing acrylamide. To avoid exposing acrylamide to co-workers, cover the weigh boat containing the weighed acrylamide with another weigh boat (similar size to the original weigh boat containing the weighed acrylamide) when transporting it to the fume hood. Transfer the weighed acrylamide to the cylinder inside the fume hood and mix on a stirrer placed inside the hood. Unpolymerized acrylamide is a neurotoxin and care should be exercised to avoid skin contact. Mixed resin AG 501 –X8 (D) (anion and cation exchange resin) is used when acrylamide solution is made, since it removes charged ions (e.g. free radicals) and allows longer storage. Some investigators store the prepared acrylamide along with this resin in the refrigerator. However, we filter them out before storage. The used mixed resin should be disposed of as hazardous waste. Manufacturer's warning states that this resin is explosive when mixed with oxidizing substances. The resin contains a dye that changes from blue-green to gold when the exchange capacity is exhausted.

3. The acrylamide solution can be stored at 4°C for one month. Acrylamide hydrolyzes to acrylic acid and ammonia. The acrylamide mixture, buffer and water can be prepared in large batches, frozen in aliquots (for greater day-to-day reproducibility) and used indefinitely (*see Ref. 12*). Remove the required amount, bring to room temperature and add the other ingredients for polymerization. However, in our laboratory we make the acrylamide solution fresh about every month when we cast our own gels.
4. We find it is best to prepare this fresh each time.
5. We find that storing at 4°C reduces its pungent smell.
6. Large well = 10 all, medium wells = 5 all, small well = 1 all. Only lay one size of wells on to the gel. Cut if necessary. Strips may be used again.
7. During the end of the run, the gel must be watched carefully in case a fire starts. Many times the gel will burn near the applicator strip. If this happens, turn off the unit. The gel can still be used if it had been focused for a long time. The bands are usually below the strip.

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Fig.1.
Gel assembly for isoelectric focusing

Fig. 2.

The membrane-gel assembly following flat bed IEF. The gel bond is shown in this figure. However, we did not use the gel bond to support the gel. The gel was directly in contact with the glass plate.

Fig. 3. Affinity immunoblotting of the patient's sera obtained at different months following first observation, showing anti-Ro 60 specific IgG clonotypes. Sera from an anti-Ro 60 negative SLE patient (Ro^-) and from two typical anti-Ro 60 positive patients (Ro^+) are shown for comparison. The pH range of the IEF gel is shown on the right.