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Analysis of antibody clonotype by affinity immunoblotting

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Summary

A sensitive and specific method to analyze specific antibody clonotype changes in a lupus patient who developed autoantibodies to the Ro 60 autoantigen under observation is described. Patient sera collected over several years were separated by flatbed isoelectric focusing (IEF) and analyzed by affinity immunoblotting utilizing Ro 60-coated nitrocellulose membrane. When the Ro 60-coated nitrocellulose was laid over the surface of the IEF gel, the antibodies present on the surface of the acrylamide gel bound the Ro antigen on the nitrocellulose. Tween-20 was used to prevent non-specific binding. The bound IgG clonotypes were detected using alkaline phosphatase conjugated anti-IgG. The patient's sera demonstrated an oligoclonal response to the Ro 60 autoantigen that increased in complexity and affinity over time.

Keywords

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

1. Introduction

Isoelectric focusing is a very useful method for investigating the heterogeneity of antibody and immunoglobulin (Ig) clonotypes (1). Antigen-specific antibody clonotype patterns can show whether changes in cell population happen during ongoing immune responses as a response to regulatory influences. It can also tell whether changes in hybridoma cell lines can occur with time (2). Previously, it was customary to study these changes by immobilizing the separated antibody clonotypes after isoelectric focusing and incubating them with radioactive antigen. In one method, radiolabeled hapten was allowed to diffuse into a gel before precipitation of Ig with sodium sulfate followed by detection of hapten-specific clonotype distribution by autoradiography (3). In another study Ig was precipitated in the gel with sodium sulfate immediately after completion of the focusing run and was crosslinked with glutaraldehyde followed by the addition of labeled antigen or anti-Ig (4). Subsequently it was shown that fixation with glutaraldehyde could decrease the antigen-binding ability of certain Ig (5). Furthermore it was shown that the previous 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. Another drawback of these methods is the excess time needed to diffuse antigen into the gel and for rinsing the unbound antigen out of the gel, which can take several days especially when using radioactive probes.

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

Here, we describe a method in which a 60,000 molecular weight Ro autoantigen was first passively immobilized on nitrocellulose membrane and placed in contact with an IEF gel that contained autoantibodies (derived from a systemic lupus erythematosus patient who developed antibodies to the Ro 60 autoantigen over time) focused according to its isoelectric point. Following diffusion mediated transfer to membrane (see **Chapters 9, 10 and 11**) the antibody clonotypes that are not antigen specific are removed by washing while the antigen-specific antibody clonotypes are detected using alkaline phosphatase conjugated anti-Ig.

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

There have been instances of some SLE autoantibodies appearing and disappearing, at times in association with specific disease manifestations, therapy or generalized clinical disease activity. For instance, 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 such as anti-Ro, on the other hand, occur in some normal subjects as well as in SLE patients before onset of disease (8), and develop only rarely during the course of SLE.

This investigation was carried out following the identification of an SLE patient who developed antibodies to the Ro 60 autoantigen after about 10 years of illness. As shown in Fig. 3 anti-Ro 60 clonality increased in complexity, and affinity to the Ro 60 antigen also increased as the response developed.

2. Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of $18 \text{ M } \Omega \text{ cm}^2$ 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 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 $0.45 \mu\text{m}$ 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- $6\text{H}_2\text{O}$ 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 $33 \mu\text{L}$ of BCIP and $66 \mu\text{L}$ of NBT to 10 mL of alkaline phosphatase buffer just before adding to membrane. Alternatively, use 1-Step™ NBT/BCIP readymade mix.
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.
13. Ro 60 autoantigen (Immunovision, Springdale, AK, USA).
14. Glass plates: Two 5" by 4" glass plates.
15. Medium binder clips (1 ¼ inch).
16. Small binder clips (¾ inch).
17. Gasket with 3 edges, about 3 mm wide, to serve as spacer between the plates.

18. LKB-2117 Multiphor apparatus for IEF.
19. Model 3000/300 power supply.
20. pH 3 and pH 10 solutions.
21. Helium gas.
22. Sample applicator strip.
23. Paper wicks.

3. Methods

All procedures are at room temperature unless otherwise specified.

1. The night before focusing cut a piece of nitrocellulose membrane according to the size that would fit a small pipet box lid (yellow tip box). Add antigen (Ro 60) at 10 µg/mL of sodium bicarbonate, pH 9.5 and incubate this with the membrane overnight with shaking.
2. 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 µL of pH 3–10 ampholytes followed by 100 µL of pH 8–10.5 ampholytes.
3. 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.
4. While the solution is degassing, set up the gel apparatus. Soak the gasket in water for few min. Mop dry with Kimwipes.
5. 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.
6. After degassing is complete, the metal end of the degassing tube is cleaned with water. To the degassed solution, add 100 µL of 10% Tween-20 and mix gently. Then add 100 µL of 2% APS. Have a Pasteur pipette ready for pouring the gel. Add 10 µL 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.
7. Turn cooling unit on and set it on 4°C in preparation for focusing.
8. After two h carefully remove one of the glass plates and gasket. The gel will remain on one of the glass plates.
9. 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).

10. 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}).
11. Prefocus by setting constant voltage 200 V for 20 min, then increase voltage to 400 V for another 20 min. Prepare samples for application.
12. 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).
13. 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).
14. Thirty min prior to end of the run, rinse nitrocellulose membrane three times with PBS, pH 7.4, shake with PBST for 30 min and rinse three times with PBS.
15. After the run is complete, transfer the focused protein from the gel to membrane. Take gel off the flatbed and remove applicator strip. Place nitrocellulose membrane between two Kimwipes and dab dry. Place the membrane over bottom half of gel above the bottom wick. The membrane may cover the applicator strip area.
16. Place gel in a Tupperware container with a moist towel, cover and place in an oven at 37°C for 20 min.
17. Take membrane off gel. Rinse with 200 mL of deionized water 2–3 times (*see* Note 8).
18. Rinse three times with PBS. Wash for 20–30 min in PBST and rinse three times with PBS.
19. Add appropriate alkaline phosphatase conjugate (10 mL) to membrane and shake for 1 h.
20. Develop bands with NBT/BCIP (*see* Fig. 3).
21. Scan the membrane and save results (12).

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. 13*). 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.
8. Rinsing the membrane with deionized water 2–3 times will help remove a bulk of the non-specific antibodies and help reduce the amount of TBST used subsequently and also reduce the number of washes. This wash helps to reduce non-specific binding of NBT/BCIP to the strip. The water, owing to its low ionic strength compared to TBST, will be able to remove contaminants much better than TBST. Water is much cheaper compared to TBST, in terms of money and

labor. Other investigators have found no reduction in detection of specific signals due to washing with water (14).

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Figure 1:
Gel assembly for isoelectric focusing

Figure 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.

Figure 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.