

A Dot-Immunobinding Assay for Monoclonal and Other Antibodies

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Received June 25, 1981

A procedure for the assay of antibodies in sera based on the application of the antigen as a spot to nitrocellulose filters is described. The method has the merit of being simpler in operation and more sensitive than comparable existing procedures. Applications for screening supernatants of hybridomas making monoclonal antibodies, and the use of such antibodies in the determination of the tissue distribution of the corresponding antigens, are described. An application for the screening of human pathological sera for multiple antibodies in one operation is also described.

Since the introduction of monoclonal antibody techniques by Köhler and Milstein (1), these precise immunochemical reagents have been applied in a wide variety of fields. Furthermore, the use of tagged antibodies provides a rapidly expanding range of clinically useful diagnostic procedures. The need to determine the specificity of an antibody against a complex mixture of protein antigens led to the development of a two-dimensional solid-phase immunoassay system for proteins electrophoretically blotted from a polyacrylamide gel onto a nitrocellulose support (2). In addition, antigen-coated nitrocellulose has been used to locate antibody-secreting cells growing in agar (3). Solid-phase systems based on nucleic acid hybridization to nitrocellulose-bound DNA have also been extraordinarily widely applied (4). A recent modification of the original Southern blot procedure is the "dot hybridization" method (5) in which, instead of being blotted from a gel, the probe is applied directly to the nitrocellulose as a spot. This allows both a considerable simplification of the procedure and the simultaneous screening of a large number of samples. We describe here an analogous protocol for antigen-antibody reactions which we, by analogy, call "dot immunobinding." We show the

application of the protocol to the screening of hybridoma clones, where large numbers of samples must be screened for a specific antibody, and as a diagnostic procedure, by which large numbers of antibodies may be assayed simultaneously.

MATERIALS AND METHODS

The principle of the assay is as follows. A diluted solution or suspension of antigen in saline is "dotted" on to a nitrocellulose filter and the dot then incubated first with the test antibody and second with a peroxidase-conjugated second antibody directed against the first antibody. After the development of the peroxidase, a positive reaction is detected as a colored dot against the white filter background. The procedure described below is that which we developed to assay antibody against synaptosomal plasma membranes (SPM)¹ from rat brain, but we have successfully used it with only trivial modifications for a large range of other antigens. The potential range of antigens to which this technique may be applied is very wide. All those we have tested so far, soluble

¹ Abbreviations used: SPM, synaptosomal plasma membranes; TBS, 50 mM Tris-HCl, 200 mM NaCl, pH 7.4; IgG, immunoglobulin G.

proteins, nucleic acids, membranes, various organelles, fungi, protozoa, bacteria, viruses, etc., have bound tightly to the filter. A typical protocol is given below.

1. *Sheet preparation.* With a pencil, draw a rectangular grid onto a nitrocellulose filter, such that each square will fit into the well of a 96-well microtiter plate, i.e., less than 4 mm per side. Alternatively, it is possible to purchase nitrocellulose sheets with a 3 × 3-mm grid already printed (Millipore Corp.). Wash the filter for 5 min by gentle agitation in distilled water and leave it at room temperature to dry. The filter should be handled throughout with forceps.

2. *Dotting.* When the filter is dry, place a small drop of antigen solution into each square. The concentration necessary will vary from antigen to antigen: for complex antigens such as membranes, 0.1–1.0 mg/ml is suitable; for a less complex mixture, the concentration can be reduced accordingly. It is often favorable to dry the filter very thoroughly at this stage, as drying can stabilize the binding. (Nucleic acid binding requires baking for 60 min at 80°C (4,6)). The antigen-dotted filter may be stored dry for several weeks at this stage without any loss of activity. The spot should be as small as possible. We find it convenient to use a 20- μ l micropipetting device. Alternatively, a Hamilton syringe can be used to apply dots as small as 100 nl. If the antigen is very dilute, it is possible to apply successive doses at the same site, taking care only that the filter is allowed to dry before each application. Leave the filter for 5 to 10 min in the cold. Wash the filter for 5 min in TBS (50 mM Tris-HCl, 200 mM NaCl, pH 7.4) and then, while the filter is still wet, cut out the individual squares. Obviously, this can be done in many ways. Where large numbers of test squares are involved, we prefer to cut individual rows with a scalpel and ruler and then cut up the rows with scissors. Place the squares face upward into the wells of a 96-well Costar tray (Costar Inc., Cambridge, Mass.). Where a range of different antigens

are to be screened, nitrocellulose strips carrying an antigen array are used. The strips can then be easily processed in an appropriate vessel, such as the troughs of a disposable "reservoir insert" (Dynatech Laboratories, Alexandria, Va.).

3. *Blocking.* To each well, add 150 μ l of 3% w/v bovine serum albumin, and 1% v/v normal goat serum in TBS, and leave the tray shaking gently for about 15 min. This treatment results in the blocking of nonspecific antibody binding sites both on the filter and on the walls of the plastic well. In our experience with a wide range of antigens, such filters can be stored dry for several months without loss of activity. The goat serum may be replaced by 10% horse serum, fetal calf serum, and probably a number of others, and bovine serum albumin may be omitted.

4. *Primary incubation.* Aspirate away the blocking solution using a Pasteur pipet connected to a suction line, and add the antibody test solution (150 μ l/well is easily sufficient, half as much will suffice). Leave the tray shaking at room temperature. The incubation time will vary from antibody to antibody. For most purposes, 2–4 h is sufficient, but for dilute solutions, an overnight incubation may be as much as 10 times more sensitive. Antibody dilutions should be made into blocking solution.

5. *Wash.* Remove the primary antibody and wash the filters for 30 min in several changes of TBS.

6. *Repeat* the blocking (step 3).

7. *Secondary incubation.* Incubate the filters for 2 h in 100 to 150 μ l of horseradish peroxidase-conjugated antiprimary species immunoglobulin. (For example, when the test antibody was raised in mouse, use peroxidase-linked goat antimouse IG, supplied by Nordic Laboratories, Tillburg, Netherlands.) Again, the concentration needed will vary from batch to batch, but 1/1000 in blocking solution is about right.

8. *Wash.* Remove the second antibody

and wash the filters for at least 30 min in several changes of TBS.

9. Development. Develop with 4-chloro-1-naphthol (Merck Inc.) and hydrogen peroxide. Chloronaphthol is prepared as a 3 mg/ml stock solution in methanol, which may be stored for 1 to 2 weeks in the dark in the cold. It should be discarded when signs of yellowing appear. Just before use, it should be diluted with 5 vol of TBS and made to 0.01% v/v in hydrogen peroxide (usually available as a 30% aqueous solution). About 100 to 150 μ l of developing solution is needed per well. Positive primary antibodies give a blue spot in 2 to 15 min. When the reaction is complete, wash the filters with distilled water and allow them to dry. Once dry, they should be stored in the dark. We have also used both *o*-dianisidine and 3,3-diaminobenzidine (both are suspected carcinogens and should be handled accordingly) as substitutes for chloronaphthol, but there is no doubt that these give substantially higher background staining with no concomitant gain in sensitivity.

RESULTS AND CONCLUSIONS

The protocol described above is extremely versatile and it is applicable to such varied problems as the detection of monoclonal antibodies and the analysis of antibodies in pathological human sera. Examples of both are given here.

Our experiments to raise monoclonal antibodies against rat SPM proteins require the quick and convenient assay of large numbers of samples. First, it is necessary to titer a panel of mice for anti-SPM antisera, then to assay the culture media from hundreds of tissue culture wells, and finally, to investigate the species and tissue distributions of those antigens against which we obtain monoclonal antibody. We describe the use of the dot immunobinding assay in all of these procedures.

The assay of five mice previously immunized with SPM is shown in Fig. 1. In each

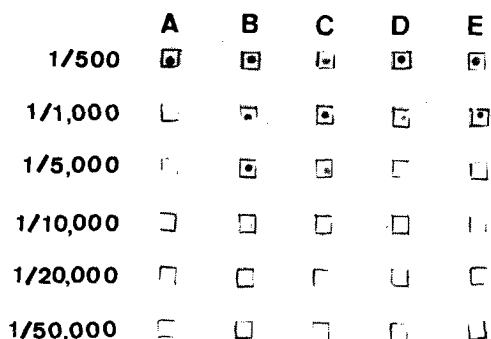


FIG. 1. A dot immunoassay for serum antibodies against SPM prepared from rat cerebellum (7). A panel of five mice were immunized twice intraperitoneally with 2 mg each of SPM with 21 days between injections. The first injection was given in Freund's complete adjuvant, the second in Freund's incomplete. Three days after the second injection, the mice were bled from the tail vein and the blood centrifuged to remove cells. A serial dilution assay of the five sera is shown. The method was as described in the text, with the SPM dotted onto the filter at 0.8 mg/ml, blocking with 10% normal horse serum 3 h incubation in the sera, and development with 4-chloro-1-naphthol. All serum dilutions were made with blocking solution. The spleens from the mice which gave the highest serum titers (1/20,000) were used to produce monoclonal antibodies.

case, the mouse serum has been serially diluted into blocking solution and then tested against a standard aliquot of SPM. It is clear that, by this method, we can rapidly identify those animals with the highest anti-SPM serum titers.

To prepare monoclonal antibody to SPM, the spleen cells of hyperimmunized mice were fused with cells from a myeloma line and the resulting somatic hybrids grown in selective medium (1). The culture media from these growing hybridoma cultures were then assayed individually for specific antibody. Such an assay for anti-SPM activity, in which both positive and negative supernatants can be seen, is illustrated in Fig. 2. In each case, the medium was taken directly from the culture well and used without dilution. The tissue distribution of one specific antigen, MIT-23, against which we have a monoclonal antibody is shown in Fig. 3. This assay is different in that, instead of testing

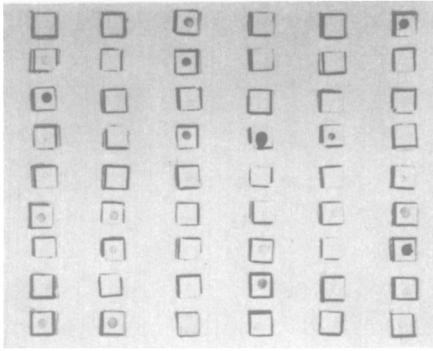


FIG. 2. A dot immunoassay for monoclonal antibodies against SPM. Hybridomas producing antibodies against SPM were derived by fusion of the spleen cells from two hyperimmunized mice (see Fig. 1) to the cells of a myeloma line (1; Hawkes *et al.*, unpublished work). The hybridomas were grown in selective medium in 480×1 -ml culture wells. After 2 to 3 weeks, most wells contained growing cells, and the media from these were taken and tested for anti-SPM activity. Fifty-four such tests with both positive and negative results are shown. The SPM was dotted at 1.0 mg/ml and the undiluted culture media incubated overnight with the filters. Development was with chloronaphthol.

a range of antibodies against the same antigen, it employs a single antibody and a range of potential antigens, in this case, crude membrane fractions from various tissues of the rat. The results show that MIT-23 is found in both the cerebellum and the cerebellar cortex of the rat brain but not in any of the other tissues tested. This assay can be quantitated approximately by spotting antigen dilution series. Conversely, it is possible to titrate the antibody and calibrate by means of an internal standard. This can be done by applying immunoglobulin of the appropriate species instead of the original antigen.

We have used a range of commercially available immunodiagnostic kits to illustrate the versatility of the technique. The test antigens and, where available, negative controls were dotted as an array onto filters as described above, the nonspecific sites blocked with 10% horse serum, and the sheets then stored dry. Individual strips were then cut from the sheet and tested with various human sera, all diluted 100-fold. Examples are

shown in Fig. 4. In separate tests, the control spot is always positive for the combination which was provided in a kit as a positive control and negative for the negative controls. This demonstrates that, in every case tried, the same specificity was obtained as would have been found using the kit as provided, and a wealth of information is obtained concerning other antibodies in the same serum. By this method, it will be possible to incorporate all established diagnostic tests, depending on the determination of individual antibodies, into one universal test.

This assay has two advantages over enzyme immunoassays which employ, for example, microtiter plates as supports. First, the amount of the antigen needed is greatly reduced because of the small spot size. In experiments with the application of various volumes, a clearly visible spot can be seen with the application of $0.1 \mu\text{l}$ of sample, the smallest volume which can reasonably be applied with commonly available equipment. The spot diameter is then 0.3 mm. The resolution limit may be well below this value.

The absolute sensitivity of the system is illustrated in Fig. 5. Various amounts of human IgG were applied in $0.5\text{-}\mu\text{l}$ spots and the color intensity from the resultant antibody reaction measured. This then gives an absolute calibration of the amount of antibody that can be detected with the system.



FIG. 3. The tissue distribution of the rat brain antigen MIT-23. A monoclonal antibody was used to assay for MIT-23 in crude homogenates from various rat tissues. In each case, the homogenate was dotted at 1.0 mg/ml. From left to right the tissues are liver (L), cerebellum (C), forebrain (F), kidney (K), thymus (T), striated muscle (S), and heart muscle (H). It is clear that MIT-23 is present both in cerebellum and forebrain but not in the other tissues tested. Experiments in which the antigen concentration in the dot was progressively reduced showed that SPM-23 could be detected in a cerebellar homogenate at $50 \mu\text{g/ml}$ but not at $10 \mu\text{g/ml}$, suggesting that the negative members of the tissue panel have less than 5% of the MIT-23 found in cerebellum.

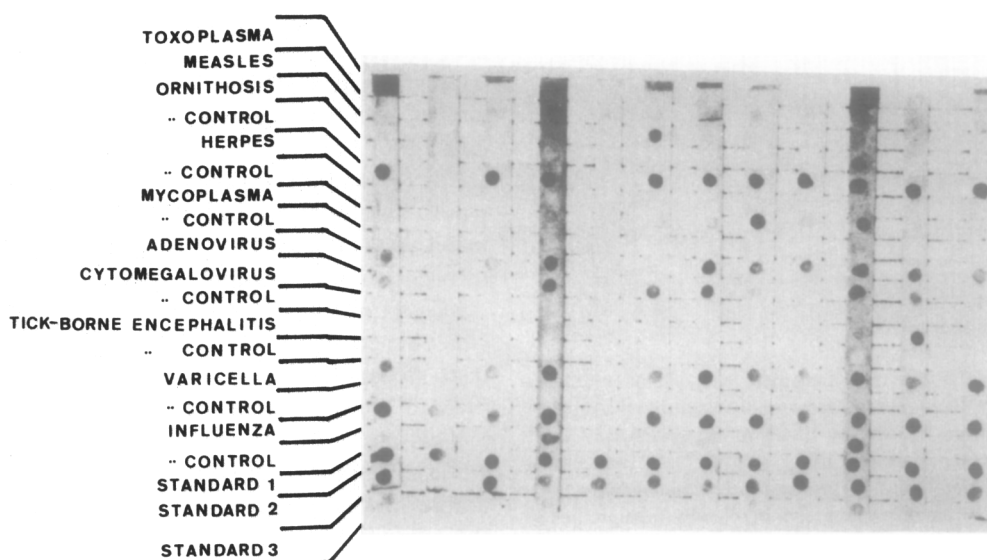


FIG. 4. Demonstration of capability of the dot-immunobinding assay as a possible clinical diagnostic tool. Antigens were obtained from the following commercially available diagnostic kits and 0.5 μ l applied to nitrocellulose in the dilution in TBS indicated. From Behring were: toxoplasma fluorescent antibody kit (undiluted), measles hemagglutination inhibition kit (1:25), ornithosis complement fixation (cf) test (undiluted), Herpes simplex I cf test (1:25), mycoplasma pneumoniae cf test (1:25), tick-borne encephalitis cf test (undiluted), varicella zoster cf test (1:5). From Flow Laboratories were: adenovirus 4 cf test (1:5) and cytomegalovirus cf test (1:5). Influenza virus was the mixed vaccine "Sandovac" from Sandoz, consisting of influenza A/Brazil/11/78, A/Bangkok/1/79, and A/Singapore/222/79 (undiluted). The negative control antigen was from a Flow Laboratories hemagglutination assay kit. The dilution giving the maximum reaction was previously determined empirically with the use of human sera known to be positive for that antigen, usually that provided with the specific kit. The internal standards 1, 2, and 3 were a normal human serum, diluted 1:500, 1:2,500, and 1:12,500, respectively, in TBS and applied as were the antigens to the nitrocelluloses. For experiments of this type, 10% decomplexed horse serum has been found to be a suitable blocking agent. Each strip was incubated overnight in a 1:100 dilution of individual human sera (also usually from one of the kits). The strips were then processed as described under Materials and Methods, except with the use of peroxidase-coupled goat antihuman IgG, and with *o*-dianisidine as the substrate (0.02% in TBS containing 0.6% H_2O_2).

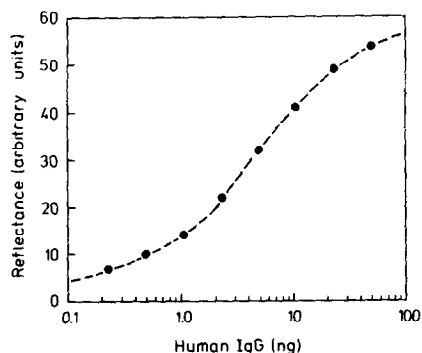


FIG. 5. Densitometric analysis with various amounts of human IgG bound to the nitrocellulose. Human IgG (Nordic) was applied in 0.5- μ l aliquots to nitrocellulose, the nitrocellulose blocked with 10% horse serum, in-

In practice, the sensitivity of detection of a particular antigen will depend on the number of determinants. Since as little as 100 pg of antibody will give a visible spot, correspondingly smaller amounts of antigen are detectable. The dynamic range of the system is also illustrated in Fig. 5. Significant changes in color intensity may be detected over 3 orders of magnitude of antibody amount.

cubated for 2 h with goat antihuman IgG, and the peroxidase color reaction performed with 4-chloro-1-naphthol as substrate. The reflectance of the spots was determined with a CAMAG (Muttentz, Switzerland) thin-layer plate scanner.

A second fundamental advantage is that the use of nitrocellulose permits the reaction to be viewed against a background which should be almost white. In this way, the discriminatory power is greater than in a microtiter plate, and it becomes much easier to detect positive reactions and to reject false positives.

ACKNOWLEDGMENTS

We thank Dr. A. Matus and Dr. D. Monard for their encouragement and advice.

REFERENCES

1. Köhler, G. and Milstein, C. (1975) *Nature* **256**, 495-497.
2. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Nat. Acad. Sci. USA* **76**, 4350-4354.
3. Sharon, J., Morrison, S. L., and Kabat, E. A. (1979) *Proc. Nat. Acad. Sci. USA* **76**, 1420-1424.
4. Southern, E. M. (1975) *J. Mol. Biol.* **98**, 503-517.
5. Kafatos, F. C., Jones, C. W., and Efstradiadis, F. C. (1979) *Nucl. Acids Res.* **7**, 1541-1552.
6. Thomas, P. S. (1980) *Proc. Nat. Acad. Sci. USA* **77**, 5201-5205.
7. Matus, A. I., Pehling, G., Ackermann, M., and Maeder, J. (1980) *J. Cell Biol.* **87**, 346-359.