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### Immunoblotting, Dot-Blotting, and ELISPOT Assays: Methods and Applications

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## Immunoblotting, Dot-Blotting, and ELISPOT Assays: Methods and Applications

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### I. INTRODUCTION

Immunoblotting (or Western blotting) involves the transfer of proteins that have been separated by electrophoresis or isoelectric focusing (IEF) from the electrophoresis gel to a membrane, to which they become bound. The bound molecules are then detected by a specific probe, usually an antibody. The advantage of immunoblotting is that it combines the resolution obtained by electrophoretic separation of mixtures of proteins in a gel with the specificity of antibodies used as probes to identify individual antigens. Proteins can be separated by any of the electrophoretic techniques available by using agarose, polyacrylamide in the presence or absence of sodium dodecyl sulfate (SDS), isoelectric focusing (IEF), or two-dimensional polyacrylamide gel electrophoresis (2-D PAGE).

Direct overlay of the gel with antibody (immunofixation) has been used to identify antigens of interest, but such methods suffer from the disadvantages of prolonged incubation times, resulting in diffusion of the bands, and consequent loss of resolution. Immunofixation is also primarily limited to agarose gel systems, since antibody molecules cannot readily penetrate polyacrylamide gels, owing to the gels' small pore size.

Because of these difficulties, the idea of transferring electrophoretically separated proteins from a gel to a membrane, where they are readily accessible to high relative molecular mass ( $M_r$ ) probes, such as antibody molecules, opened up a new vista for electrophoretic analysis of proteins. The method of transfer is termed *blotting*, since the pattern of bands on the membrane is an exact replica of the pattern in the original gel. The Southern-blotting method for analysis of DNA was named after Ed Southern; hence, analysis of RNA molecules by a similar technique was called Northern blotting, and analysis of proteins by transfer to a membrane and detection by antibody became known as Western blotting or immunoblotting. Dot-blotting, slot-blotting, and line-blotting are variants of this in which antigens are applied directly to a membrane without prior separation and identified in the same way. The amount of antigen present in the mixture can be measured quantitatively. ELISPOT assays use similar principles to measure the number of cells secreting specific antibody or other proteins.

Many adaptations have been developed and applied to a wide variety of fields of research and clinical applications. In this chapter I shall discuss the basic techniques and principles of immunoblotting, dot-blotting, and the ELISPOT assay, and describe a selection of some of their

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applications. Earlier methods and applications have been described in a previous review of the subject (Stott, 1989).

## II. PRINCIPLES

The aim of immunoblotting is to make a replica of the pattern of bands of a mixture of proteins, after separation in an electrophoresis gel, on the surface of a thin membrane so that the antigens are readily accessible to specific antibody or other probes. In the ideal blotting system, therefore, the blot would be an exact replica, qualitatively and quantitatively, of the electrophoresis gel, with no diffusion and with all protein bands represented in the same proportion as on the original gel in their native conformation. The ideal membrane would bind all proteins irreversibly, independently of size, charge, and sequence. Once bound, the antigen(s) of interest are detected by antibody, usually in two layers. The first layer is antibody specific for the antigen to be identified; the second layer is the detection system, which may be anti-immunoglobulin or other molecules that bind to the first antibody. The second layer molecules are conjugated to a label (radioisotope, enzyme, or other) that enables the bands containing the antigen to be identified. This may include an amplification system to increase sensitivity.

## III. IMMUNOBLOTTING METHODS

### A. Equipment

Several types of electrophoretic blotting apparatus that perform reasonably efficiently are available commercially and fall into two broad categories: (1) total immersion systems, in which the gel and membrane are immersed in buffer vertically in a tank; (2) the semidry system, in which the gel and membrane are laid horizontally between sheets of absorbent paper, moistened with buffer. The commercial equipment is, for most purposes, perfectly satisfactory, but it is also possible to build a transfer tank quite cheaply to suit one's own requirements. Features requiring special consideration to maximize efficiency and uniformity of transfer, with efficient binding of proteins to the membrane, have been discussed previously (Stott, 1989; Gershoni et al., 1985).

The total immersion system uses a plastic cassette that holds the gel and membrane in a "sandwich." The cassette is inserted vertically into the tank and fully immersed in buffer. A space between the sandwich and electrodes allows free release of the large quantities of hydrogen and oxygen liberated at the electrodes by the high current. Naked flames should not be allowed near the apparatus for this reason. Where plate electrodes are used they may be placed in direct contact with the cassette, thereby increasing the voltage gradient and efficiency of transfer, in which case, spacers are glued to the surfaces in contact with the electrodes to allow free escape of gas (Stott et al., 1985). It is important to eliminate air bubbles trapped between the membrane and gel or paper and gel as these obstruct the passage of current and, hence, transfer of proteins. Air bubbles can be seen through the translucent wet membrane and removed by gentle stroking. Transfer is carried out at room temperature, but at high currents the gel should be cooled by circulating tap water through cooling coils owing to the heat generated by the current.

Horizontal blotting systems are simple to use and economical on buffer. They dispense with the tank altogether, as the current is conducted through layers of chromatography paper saturated with buffer. The electrodes are usually plates made of graphite, platinum-coated titanium, or stainless steel. Several layers of chromatography paper moistened with buffer are placed on the horizontal anode plate, the gel sandwich is then positioned on the wet paper, with the membrane facing the anode and covered with additional layers of paper soaked in buffer, followed by the cathode plate. This system has the advantage that the gel sandwich is set up in situ and several

gels can be blotted simultaneously by placing them on top of each other; passage of proteins through the membrane of one sandwich to the next being prevented by interposing sheets of dialysis membrane between the layers. Much smaller quantities of buffer are used compared with the vertical system and no cooling is required, owing to the low power consumption. A possible disadvantage is the danger that gas generated at the surfaces of the electrode plates will become trapped and inhibit or distort the transfer of proteins, but this does not appear to be a serious problem in practice.

## B. Transfer of Proteins from Gel to Membrane

The simplest method of transferring protein from a gel to a membrane is by inducing a flow of solvent from the gel through the membrane by capillary attraction. This is achieved by placing a membrane on top of the gel, followed by chromatography paper and a pile of paper towels on top of the membrane. Fluid is drawn by capillary attraction through the membrane into the paper towels, and proteins are trapped in the membrane. Transfer is aided by placing wet chromatography paper under the gel, as in the original Southern technique for DNA blotting, but this is not essential, and acceptable transfer efficiencies can be obtained by omitting the buffer source and allowing the gel to dry to a thin layer during blotting. Capillary blotting is very inefficient with polyacrylamide gels, owing to obstruction of the movement of protein molecules by the small pores of the gel; prolonging the transfer period allows diffusion of the bands with loss of resolution. It works best with agarose gels, which have large pores that do not hinder the movement of protein molecules. Efficient transfer can be achieved in about 90 min–2 h, and acceptable results can be obtained in as little as 10 min, with some sacrifice of efficiency. Advantages of capillary blotting are simplicity of operation and ease of handling, since the gel need not be removed from its supporting plate or plastic film.

Electrophoretic transfer is preferred for polyacrylamide gels, as it is faster, and complete transfer can usually be obtained. A wet membrane is placed on one side of the gel, ensuring uniform contact over the whole area. The gel and membrane are then sandwiched between pads of foam rubber, scouring pads, or chromatography paper, saturated with the transfer buffer. The sandwich is supported between plastic grids held vertically in a tank of buffer and a potential gradient applied across the gel, usually for about 2 h. Problems that can arise are incomplete transfer because of too short a transfer period, voltage too low, pH of the transfer buffer too low, proteins aggregated in the gel, and absence of SDS in the gel. Uneven transfer may be due to inappropriate geometry of the electrodes or cassette, trapped air bubbles, high- $M_r$  proteins moving more slowly than low- $M_r$  proteins, or proteins that do not bind or bind poorly to the membrane. It is advisable to test the efficiency of transfer when blotting proteins for the first time, by staining both gel and membrane (see Sec. III.C.5). If incomplete binding to the membrane is suspected, a second membrane can be placed behind the first and stained for protein.

### 1. Type of Gel

In principle, immunoblotting can be performed using any type of gel used for protein electrophoresis. Proteins can be transferred from agarose gels by capillary blotting, but electroblotting is recommended for polyacrylamide gels for the reasons given earlier. Two-dimensional gels can be treated in the same way as one-dimensional polyacrylamide gels. Polyacrylamide isoelectric focusing gels should be preequilibrated with transfer buffer containing 1% SDS and 20% glycerol, instead of methanol (to prevent swelling of the gel), since the focused proteins are at their isoelectric points and do not transfer efficiently without preequilibration (Stott and McLearn, 1986). If it is desired to maintain the proteins in their native conformation, the SDS may be omitted, but the pH of the transfer buffer should be increased to pH 8.8, and transfer may be less efficient.

Polyacrylamide gels can also be run under nondenaturing conditions, and this is particularly useful when epitopes are sensitive to denaturation or when a molecule is to be detected after blotting by its enzymic activity (Van Seuning and Davril, 1990; Brahimi-Horn et al., 1991).

## 2. Membranes

A variety of different kinds of membrane are now available for immunoblotting, but nitrocellulose is still the most widely used. The physicochemical basis of binding of proteins to nitrocellulose is believed to be largely by hydrophobic interactions. Ionic interactions are unlikely to be involved to a large extent, since the nitro group is not charged. This is borne out by the observation that nonionic detergents, such as Triton X-100 or Nonidet P40, can elute up to 90% of bound protein, which should be taken into account if washing buffers containing detergents are used to reduce nonspecific binding. Hydrogen bonding with the nitro group also probably contributes to the binding energy. The theoretical basis of the interaction of proteins with nitrocellulose membranes has been discussed by Van Oss et al. (1987).

Fixation of proteins to the membrane can be used to prevent elution during washing and incubation steps. Acid and alcohol, glutaraldehyde, chemical crosslinking, and ultraviolet irradiation, all have been used (Gershoni and Palade, 1982; Kakita et al., 1982; Jahn et al., 1984; Faye and Chrispeels, 1985), but the epitopes of many proteins are sensitive to such treatment and may no longer be detectable by antibody, so each antigen-antibody combination should be tested before being subjected to this type of treatment. Ikegaki and Kennett (1989) fixed the primary antibody-antigen complexes to the nitrocellulose membrane with glutaraldehyde, after they had been formed, avoiding the problem of altering epitope structure. A further disadvantage of nitrocellulose is its relatively low protein-binding capacity, in the region of 80  $\mu\text{g}/\text{cm}^2$  (Gershoni and Palade, 1982). Membranes containing mixtures of nitrocellulose and cellulose acetate (e.g., the Millipore HAWP series) have even lower capacities, so it is preferable to use the pure form.

Several other types of membranes are now commercially available, such as nylon and polyvinylidene difluoride (PVDF) (e.g., Immobilon-P, Millipore). These membranes are extremely tough and do not crack when dry, unlike nitrocellulose. They also have a much higher protein-binding capacity, and proteins are not so easily washed off (Tovey and Baldo, 1989). Cationic nylon membranes, although they have a very high affinity for proteins, bind many proteins nonspecifically, resulting in high backgrounds, even after blocking; most of the commonly used protein stains (Coomassie blue, amido black, Ponceau S) also bind to these membranes, although alternative-staining methods have been devised (see Sec. III.C.5 on general protein stains).

The ideal membrane would bind antigens irreversibly (e.g., by the formation of covalent bonds). Several attempts have been made to produce such membranes, but problems were encountered because of instability in the transfer buffer. One such membrane was produced commercially, but has since been withdrawn. Horner et al. (1989) successfully activated nitrocellulose membranes with cyanogen bromide to identify allergens reactive with IgE antibodies, but this has not been adopted by others. Desvaux et al. (1990) also used CNBr-nitrocellulose to blot antibodies against grass pollen allergens from agarose IEF gels.

## 3. Transfer Buffers

The most widely used buffer for electrophoretic transfer of proteins is still 20% methanol/Tris, glycine, pH 8.3. The methanol prevents swelling of the gel and enhances the binding of proteins to the nitrocellulose membrane, although it can reduce the efficiency of elution of protein from the gel. For isoelectric-focusing gels we use Tris-glycine buffer at pH 8.8 to ensure that it is well above the pI of the proteins. Buffers containing glycine should not be used with membranes that bind covalently to proteins, since the binding sites on the membrane would be blocked by the primary amino group of the glycine. Other buffers, such as 0.025 M sodium phosphate or 0.01 M

sodium borate, can be used instead. If methanol is omitted the gel should be preswollen in the same buffer before transfer.

#### 4. Antigen Denaturation

Proteins separated by SDS-PAGE are invariably denatured by contact with SDS and heating during sample preparation, which can prevent their recognition by antibodies. Epitopes may also be lost by unfolding and separation of polypeptide chains after reduction with 2-mercaptoethanol or dithiothreitol. Urea and methanol can also cause denaturation. Occasionally, this is irreversible, and the antigen can no longer be detected, but usually antigenicity can be regained.

Many antibodies still recognize protein antigens after blotting from denaturing gels, possibly because SDS is stripped off the protein during transfer, allowing renaturation to occur, and because some epitopes are not conformation-dependent. Monoclonal antibodies are particularly susceptible to loss of binding activity through antigen denaturation, since they normally recognize only one epitope. If a problem is suspected, attempts can be made to renature the protein on the membrane by incubating in buffer containing 4 *M* urea or a nonionic detergent, such as Nonidet P40, although possible elution of antigens from the membrane by such treatment should be taken into consideration (see Sec. III.C.1). Wedege et al. (1988) tested 14 different detergents and concluded that ionic and zwitterionic detergents, with alkyl chain lengths of at least ten carbon atoms, are required. Effectiveness of renaturation was proportional to chain length. Van Dam et al. (1990) concluded that incubation of blotted nuclear proteins with a nonionic detergent is necessary to renature the proteins, despite problems with reproducibility owing to variable elution.

Denaturation can also be avoided simply by omitting the SDS from the sample and gel, which will give a separation pattern dependent on charge as well as  $M_r$ , or by using an alternative system, such as isoelectric focusing, in which proteins are separated according to pI.

### C. Detection of Antigen

#### 1. Blocking

Before probing for the antigens of interest, it is essential to block all protein-binding sites on the membrane after transfer to prevent nonspecific binding of the probe. The blocking agent and conditions used will depend on the type of membrane, the probe, and the sensitivity of the system under investigation. Many different blocking agents have been used, bovine serum albumin (BSA), serum, and milk being among the most popular. The blocking agent must not cross-react with the probe and, when blocking with serum, it is advisable to use serum from the same species as the final antibody if possible (e.g., if the primary probe is a mouse monoclonal antibody and the secondary probe is rabbit antimouse immunoglobulin, normal rabbit serum would be a suitable blocking agent). While having the merit of being inexpensive, gelatin is not usually an effective blocking agent, although good results are said to have been obtained in some systems. Powdered milk or casein give very good, low backgrounds, are inexpensive, and readily available, but may inhibit binding of antibody to some antigens (personal observation), and significant amounts of protein are eluted if prolonged blocking times are used (e.g., 25% of bound protein is lost when nitrocellulose is incubated in 5% milk for 24 h; DenHollander and Befus, 1989). Craig et al. (1993) found that about 6% of human sera give high backgrounds with milk and other animal proteins, including BSA, apparently owing to natural antibodies against these proteins. The problem was eliminated by blocking with human serum albumin (HSA).

Nonionic detergents have also been used as blocking agents and have the advantage of being inexpensive and readily available. The membrane can be stained after blocking, which is more difficult with protein blockers, but possible in some circumstances (see Sec. III.C.5). Mohammed

and Esen (1989) claimed that a blocking step is unnecessary if Tween-20 is included in the washing and dilution buffers, although this may not apply to all systems. Detergents have the severe disadvantage of causing loss of protein by elution from the membrane; losses can be as high as 80–90% with Nonidet P-40, Triton X-100, and Tween-20. Despite this drawback, good results were obtained by Tovey et al. (1989), who found that Tween-20 improved binding of IgE antibodies to house dust mite allergens, and van Dam et al. (1990) found that nonionic detergents improved results with antinuclear antibodies, although problems with reproducibility were encountered. Improved antibody binding may, in part, be due to removal of SDS and renaturation of protein by detergent micelles.

Other factors may also affect nonspecific staining of blots. Thean and Toh (1989) found that incubation at 4°C reduced background staining, independently of the type of blocker used.

## 2. Primary Probes

Protein molecules blotted on to a membrane are most commonly probed with a specific antibody, which may be in the form of antiserum, affinity-purified antibody, or monoclonal antibody. Any probe that binds specifically to the protein under investigation can be used in place of antibody (e.g., lectins for detection of glycoproteins, DNA or RNA for nucleic acid-binding proteins, hormones for detection of hormone receptors). The primary probe may be labeled directly (see Sec. III.C.4) or a secondary probe may be used.

The primary antibody or other reagent is diluted in blocking solution to prevent nonspecific adsorption to the membrane, the optimal dilution, incubation time, and temperature being determined for the system under investigation (see Sec. III.E). The membrane is immersed in sufficient overlay solution, containing the probe, to just cover it and incubated on a rocking machine at 15–60 cycles/min, usually for 1 h at 37°C, 2 h at ambient temperature, or overnight at 4°C. Longer incubations may sometimes be necessary (e.g., for IgE antibodies). If the probe is in short supply, it can be applied to a narrow strip of chromatography paper or cellulose acetate, which is then laid over a narrow strip of the blotted membrane, protein side uppermost, in a shallow trough cut in a Perspex block (5 mm deep). The block is covered with a glass plate to prevent evaporation and incubated as before without rocking. Washing and probing with a second-layer antibody are performed in deeper troughs (10-mm deep) cut in a separate block. A large number of membrane strips can be probed with different antibodies in this way. A micromethod for probing antigens on very small strips of nitrocellulose (2–3 mm wide) has been described by Nghiêm (1988) who used the technique to screen hybridoma supernatants taken directly from multiwell tissue culture plates. As little as 50  $\mu$ l of culture fluid can be assayed for antibody activity in this way.

Whatever the nature of the primary probe, unbound probe is rinsed off with buffer, and the membrane is thoroughly washed in the same buffer, normally without a blocking agent. This is carried out on a rocker or shaker with four to six changes of ice-cold buffer, approximately 10 min per change. If high nonspecific binding of the probe is a problem, it may be necessary to add a nonionic detergent to the wash buffer (e.g., Triton X-100 or Tween-20, at concentrations from 0.05–1%), but elution of proteins from the membrane may be an undesirable side effect, especially at higher concentrations, as described earlier (see III.C.1).

## 3. Secondary Probes

A secondary probe can be used, either to amplify the signal or simply for convenience. It is especially useful when several different primary probes are used, since one secondary probe can be labeled and used for all of them. The secondary probe can be anti-immunoglobulin or protein A (from *Staphylococcus aureus*). If anti-immunoglobulin is used, it is essential to ensure that it does not cross-react with components of the blocking buffer or the antigens under

investigation. Protein A reacts with many species of IgG and, therefore, cannot be used if serum is included in the blocking buffer, or if the antigen preparation contains immunoglobulin. Protein A does not bind equally well to all types of IgG; for example, it binds well to human IgG1, 2, and 4; mouse IgG2a and 2b; rabbit, rat, guinea pig, and pig IgG; but poorly or not at all to human IgG3; mouse IgG1 and 3; chicken, sheep, and goat IgG; and IgM, IgA, IgD, and IgE of any species. Streptococcal protein G, which has a broader specificity than protein A, is an alternative.

Advantages of the double-layer technique are that (1) the same labeled secondary probe can be used for many primary antibodies of different specificities, as well as suitable negative controls, without the necessity of purifying and labeling each one. (2) A second-layer antibody enhances the signal, since more than one molecule of anti-immunoglobulin can bind to the primary antibody, resulting in increased sensitivity. (3) It avoids modifications of the primary antibody by radiolabeling, conjugation, or other, which may lead to nonspecific binding or loss of specificity.

#### 4. Labeling Methods

*Radioisotopes.* Antibodies or other probes radiolabeled with  $^{125}\text{I}$  are often used in immunoblotting because of their high sensitivity and flexibility, despite the inconvenience of handling them. They can readily be prepared in the laboratory by the chloramine-T reaction or the iodogen method (Fraker and Speck, 1978). Other isotopes can also be used. For maximum sensitivity,  $^{131}\text{I}$  may be preferred, as it produces a much denser image than  $^{125}\text{I}$  owing to greater efficiency of trapping of the  $\beta$ -radiation by x-ray film, compared with the  $\gamma$ -radiation emitted by  $^{125}\text{I}$ . The shorter half life of  $^{131}\text{I}$  (8 days) is a disadvantage, as it cannot be stored for long periods. Other isotopes, such as  $^{35}\text{S}$  and  $^{14}\text{C}$ , have also been used, but require longer exposure.

Fluorography, in which the image is enhanced by means of an intensifying screen or fluor, is widely used for detection of high energy  $\beta$ - and  $\gamma$ -emitters. The x-ray film is sandwiched between the membrane and intensifying screen and placed in a cassette or between glass plates in a black plastic bag at  $-70^\circ\text{C}$ .  $\beta$ -Particles or  $\gamma$ -rays that are not trapped in the emulsion pass through the film to the screen and excite the fluor, which emits photons. These activate the silver halide crystals in the emulsion, resulting in a greatly enhanced image; 30- to 40-fold for  $^{125}\text{I}$ . For low-energy  $\beta$ -emitters such as  $^3\text{H}$ ,  $^{14}\text{C}$ , or  $^{35}\text{S}$ , the membrane can be impregnated with diphenyl oxazole (PPO) or one of the commercial fluorography solutions, such as EN $^3$ Hance (NEN, Boston, Massachusetts) or Amplify (Amersham International, Amersham, UK).

Precise alignment of the x-ray film with the membrane can be obtained by marking two corners of the membrane and the center of the opposite side with spots of radioactive ink. The latter is prepared by addition of a small amount of any  $^{14}\text{C}$ -labeled compound to ordinary fountain pen ink or India ink to about 40–80 kBq/ml. If the membrane is later stained (see Sec. III.C.5), this allows unequivocal identification of the tracks if some of them do not contain detectable bands and can also be used for precise localization of a radioactive band relative to a stained band on the membrane.

The advantages of using radioisotopes are their high sensitivity, the ability to obtain several exposures from the same blot for optimal sensitivity and resolution, and the ability to quantitate the image by scanning densitometry. The disadvantages are that precautions must be taken during handling and disposal of radioisotopes, and exposure times can be very long, from a few hours to 2–3 weeks.

*Enzyme-Conjugated Probes.* Enzyme-conjugated antibodies are commonly used for immunoblotting, their main attractions being ease of handling and rapid development of an image. Horseradish peroxidase (HRP)- and alkaline phosphatase-conjugated antibodies are available commercially or may be prepared by coupling with glutaraldehyde. Some inactivation of the enzyme or antibody may take place, so the product should be tested for both enzyme



and antibody activity (e.g., by dot blotting; see Sec. IV). The image is developed by means of a chromogenic substrate, such as diaminobenzidine or 4-chloro-1-naphthol (for horseradish peroxidase) or 5-bromo-4-chloroindolyl phosphate (BCIP) for alkaline phosphatase. Young (1989) recommended a mixture of 4-chloronaphthol and diaminobenzidine, which gave a black product with a sensitivity of 0.1-ng protein in a dot blot. The signal can be enhanced by overlaying with anti-immunoglobulin, followed by peroxidase-antiperoxidase (PAP) complexes. Peroxidase activity is inhibited by azides, so sodium azide should not be included in the wash buffer, although it can be added to the blocking buffer, provided it is washed out before addition of substrate.

Advantages of enzyme-conjugated antibodies are ease of handling and storage (no problem of decay if stored in aliquots at  $-20^{\circ}$  or  $-70^{\circ}\text{C}$ ) and rapid development of the color (minutes instead of days). Sensitivity can be as low as 0.1–10 ng of antigen per band, but the use of radioisotopes with fluorography is usually more sensitive. Some disadvantages of enzyme conjugated probes are the following:

1. Certain horseradish peroxidase substrates (e.g., 3,3-diaminobenzidine and *o*-dianisidine) may be carcinogenic; 4-chloro-1-naphthol and tetramethylbenzidine are preferred, since they are believed to be noncarcinogenic. Tetramethylbenzidine is also more stable than diaminobenzidine (Brand et al., 1990).
2. Once the color has developed, it is not possible to intensify or reduce it to improve the image.
3. The color fades on drying and, with horseradish peroxidase, in light. Fading caused by drying can be reversed simply by wetting the membrane; loss of the image from bleaching can be prevented by storing in the dark.
4. Artifactual bands may be seen because of nonspecific interactions of the enzyme with certain proteins. Such artifacts can arise whatever labeling method is used, and a control strip incubated with a nonspecific primary antibody, or antibody of irrelevant specificity, should always be included.

Horseradish peroxidase and alkaline phosphatase can be detected by reactions in which light is generated and an image produced on x-ray film. A blot with bound HRP-conjugated antibodies can be developed with  $\text{H}_2\text{O}_2$  and luminol in the presence of an enhancer, such as 4-iodophenol or firefly luciferin and 4-methylumbelliferone (Laing, 1986; Hauber and Geiger, 1987; Schneppenheim and Rautenberg, 1987; Leong and Fox, 1990). The luminol is oxidized to a chemiluminescent product, with emission of photons, which are detected by exposing the membrane to x-ray film and developing as for autoradiography. Exposure times are extremely short (from 1 s to 10 min), and multiple exposures can be made from the same blot, which is not possible with chromogenic substrates. The sensitivity of the iodophenol-luminol system is about 100 times greater than the color reaction, and is similar to radioactivity (Schneppenheim et al., 1991). The luciferin-methylumbelliferone method is less sensitive. Xu et al. (1992) were able to detect as little as 2 pg/ml of low- $M_r$  IgM in human sera by chemiluminescence; Coloff et al. (1992) exploited its high sensitivity to identify antigens of house dust mites (*Dermatophagoides pteronyssinus* and *Euroglyphus maynei*) bound by IgE antibodies in sera from patients with atopic dermatitis.

Alkaline phosphatase-conjugated antibodies can also be used to generate chemiluminescence by hydrolysis of d-luciferin phosphate to luciferin, which then releases energy in the form of photons in a reaction with ATP and oxygen, catalyzed by firefly luciferase (Hauber et al., 1989): 5–50 fg of IgG could be detected with a photon-counting camera.

**Fluorochromes.** Antibodies and other probes can be labeled with fluorescent dyes, the blot being viewed and photographed under UV light. Dansyl chloride, fluorescein, and a europium chelate, all have been used (Abuharfeil et al., 1991; Bower and Chantler, 1991; Diamandis et al.,

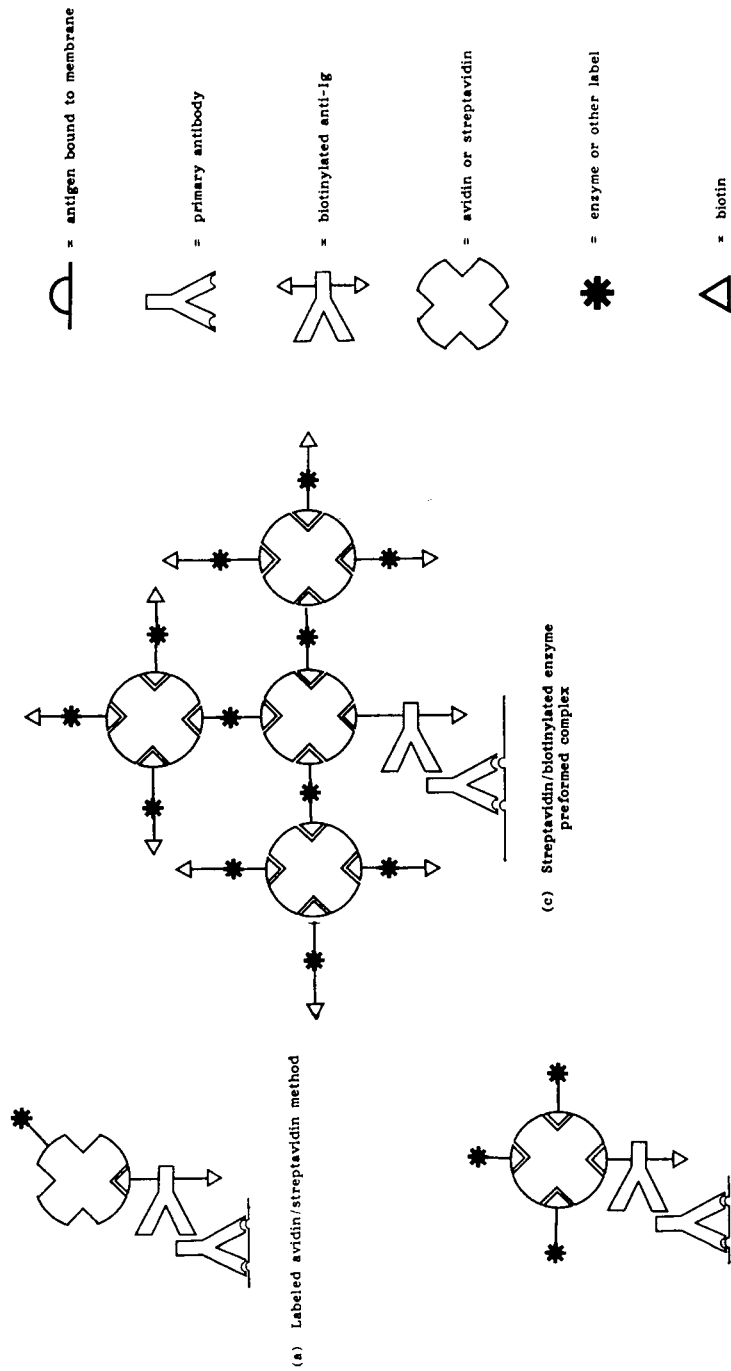
1992). The sensitivity of dansyl-labeled antibodies is similar to horseradish peroxidase, but they are simpler to prepare and are not inhibited by sodium azide, commonly added to buffers as a bacteriostatic agent.

**Gold-Labeled Antibodies.** Antibodies labeled with colloidal gold have also been used for immunoblotting and dot-blotting (qv, Brada and Roth, 1984; Hsu, 1984; Kunakorn et al., 1991; Petchclai et al., 1991). Gold-labeled antimouse IgG, antirabbit IgG, and protein A are commercially available (Janssen Life Sciences Products, UK; Sera-lab, UK; BioCell Research Laboratories, UK). A gold-streptavidin conjugate has been used with biotinylated antihuman IgG to spectrotyping IgG in cerebrospinal fluid samples (Nespolo et al., 1989). Particle sizes of 15–40 nm are suitable for staining blots. Pink bands are produced, which can be enhanced by developing with a silver stain. Sensitivity is greater than the peroxidase detection systems and can be as low as 50 pg of antigen (Moeremans et al., 1984; 1985), but the technique has not been widely used for immunoblotting. Gold or silver staining has been used to intensify conventional peroxidase-diaminobenzidine-stained blots (Iida et al., 1990).

**Biotin-Streptavidin.** As an alternative to labeled antibody, the very high affinity of biotin for the multivalent proteins avidin and streptavidin has been exploited. Any of the labeling methods described in the foregoing can be employed, although enzyme-conjugated avidin or streptavidin are most commonly used. Three different versions are available (see Fig. 1). In the first method (a), the membrane, coated with primary antibody, is overlaid with biotinylated anti-immunoglobulin, followed by labeled streptavidin or avidin, which binds tightly to the anti-immunoglobulin. Alternatively, unlabeled streptavidin may be allowed to bind to the biotinylated anti-immunoglobulin, followed by biotinylated enzyme so that the streptavidin forms a bridge between the two (b). In the third method (c), a complex of streptavidin and biotinylated enzyme is allowed to bind to the biotinylated anti-immunoglobulin. Problems have been encountered with high backgrounds owing to nonspecific binding, especially by avidin. Streptavidin is said to give better results, as it is not charged at physiological pH and sensitivity is claimed to be higher than for standard antibody-enzyme conjugates. The relative merits of digoxigenin-antidigoxigenin (a similar haptin-antihaptin system) and biotin-avidin or streptavidin are discussed by Kessler (1991).

**Multiple Probing.** To identify several antigens in the same sample, blots can be probed with two or more antibodies of differing specificity. For most purposes it is adequate to run the same sample in a single, wide slot and cut the membrane into strips, each of which can then be developed separately. However, occasionally, it may be desirable to probe the same track with different antibodies (e.g., when it is suspected that two molecules run very close together, or when immunoblotting a two-dimensional gel). Multiple blots can be made by replacing the membrane at intervals during blotting and probing each with a different antibody. Desvaux et al. (1990) made reproducible multiple capillary blots from agarose gel in this way. A disadvantage of this method is the low recovery of protein on each blot. If transfer efficiency is increased by prolonging the blotting time, reproducibility is likely to be compromised. Blots can also be probed sequentially, the preceding probe being eluted before addition of the next, but the elution buffer may remove antigen from the membrane as well as the probe. Hobart et al. (1992) synthesized a biotin derivative, *N*-biotinyl penicillamine, which could be coupled to antibodies by the cleavable linker SPDP [succinimidyl 3-(2-pyridyl)thio] propionate]. After development with <sup>125</sup>I-streptavidin and fluorography, the complexes could be cleaved by reduction with 2-mercaptoethanol and the blot reprobed using the same protocol.

If different detection systems are used for each antibody, the same membrane can be probed by different antibodies, without intermediate elution or cleavage steps. By applying antibodies conjugated to different enzymes and developing with different substrates, double- or even triple-



(b) Avidin/streptavidin bridge

**Figure 1** Diagrammatic representation of three methods of detection of antigen bound to a membrane using biotin and avidin or streptavidin. In all three cases the secondary probe is biotinylated anti-immunoglobulin. The complexes of membrane-bound antigen, primary antibody, and biotinylated anti-immunoglobulin are detected using (a) labeled avidin or streptavidin; (b) avidin or streptavidin, followed by biotinylated enzyme, which binds to the free binding sites on the avidin or streptavidin (the bridge method); or (c) a preformed complex of avidin or streptavidin and biotinylated enzyme is added and the remaining free binding sites bind to the biotinylated anti-immunoglobulin. Horseradish peroxidase is most commonly used to generate a visible signal, but other labeling methods can be used. (From Stott, 1989.)

color staining is possible, allowing the possibility of distinguishing between different antigens or isotypes of the same substance in the same mixture of proteins (Geysen et al., 1984; Lee et al., 1988). Lee et al. were able to distinguish between three different forms of interferon on the same blot by overlaying with antibodies of different species plus horseradish peroxidase- or alkaline phosphatase-conjugated anti-immunoglobulins specific for each species of primary antibody. The color was developed using two or even three different substrates, resulting in different-colored bands. Steffen and Linck (1989) used a similar approach to probe antigens blotted from 2-D gels with a combination of horseradish peroxidase-luminescence, alkaline phosphatase with a chromogenic substrate, and gold-conjugated antibody enhanced with silver.

## 5. General Protein Stains

Nitrocellulose membranes can be stained with one of the standard protein stains, such as amido black 10B (also known as amido schwarz or naphthalene blue/black), aniline blue black, Ponceau S, or fast green. Coomassie brilliant blue R binds to nitrocellulose and, therefore, gives high background staining. These anionic dyes are not suitable for positively charged nylon membranes, to which they bind strongly. Staining with silver or colloidal gold is very sensitive, especially when the latter is enhanced by silver staining (Moeremans et al., 1985; Merrill and Pratt, 1986). A very simple and sensitive general protein stain for nitrocellulose makes use of the binding of colloidal carbon (India ink) to proteins in the presence of a nonionic detergent, such as Triton X-100 or Tween-20 (Hancock and Tsang, 1983). Pelikan fount India ink gives the best results, and we have found that it is even possible to stain the membrane after blocking and fluorography or autoradiography. Although the background stains gray because of the blocking proteins bound to the membrane, the tracks are readily discernible, making it possible to identify the precise location of a particular band on the autoradiograph. Staining can be used to locate specific antigens in relation to nonantigenic molecules. Shen et al. (1990) prestained their 2-D blots with fast green and recorded the result on film to locate the antigens of interest in the complex 2-D profile.

Small polypeptides that do not bind conventional protein stains can be detected by soaking the membrane in 50% ethanol/H<sub>2</sub>O for a few seconds before chlorination and development with starch/KI solution (Rydon and Smith, 1952; Stott, D.I., unpublished observations). Unlike most protein stains, which require ionic interactions, this method will identify any molecule containing a peptide bond.

Proteins can be stained in the gel with CuCl<sub>2</sub> or ZnCl<sub>2</sub> before transfer. The staining does not appear to interfere with either the transfer process or recognition by antibodies (Wang et al., 1989; Seeber and Lucius, 1991). Molecular mass standards, labeled with a radioisotope, fluorescence, or biotin can be applied to the gel before electrophoresis and blotted on to the membrane. Iodination of *M<sub>r</sub>* marker proteins, in our experience, results in heavy labeling of one or two components and very weak labeling of the rest. Therefore, it is necessary to label the proteins separately and mix them in equal proportions relative to radioactivity. Biotin-conjugated standards (available from Bio-Rad, USA) can be identified by one of the methods described in Sec. III.C.4.e.

## D. Quantitation

The amount of antigen present in bands blotted on to a membrane can be measured by using a radiolabeled probe and scanning the autoradiograph or fluorograph with a transmission densitometer. Most densitometers give a direct readout of the area under each peak, but careful attention should be paid to the baseline, which can cause significant errors, especially if it is variable, as often happens. Peak area is plotted against concentration using standards, which

should give a linear relation over the range to be measured. Other possible causes of error are incomplete transfer from gel to membrane or inefficient retention of antigen by the membrane. Multiple antigens in the same sample can be measured by probing with a mixture of antisera (Schilling and Aletsee-Ufrect, 1989).

Colored bands on a membrane can also be quantified, either by reflectance densitometry or by transmission densitometry after soaking the membrane in an organic solvent, such as xylene, liquid paraffin, or microscope immersion oil, to make it transparent. Alternatively, bands can be excised, solubilized and read spectrophotometrically (Gershoni, 1988; Uhl and Newton, 1988). Fluorescent bands can be measured by scanning with a time-resolved fluorometer (Diamandis et al., 1992). The signal produced on x-ray film by chemiluminescence can be measured by scanning densitometry. Heinicke et al. (1992) were able to detect less than 10 fmol of protein, a sensitivity comparable with  $^{125}\text{I}$ -protein A.

One of the advantages of measuring protein concentration from immunoblots is that the separation process removes interfering substances (e.g., cross-reacting antigens or enzyme inhibitors) from the antigen to be measured. It also makes it possible to separate different forms of the same antigen (e.g., isotypes or precursor molecules). If this is not required, antigen concentration can be measured quickly and simply by dot- or slot-blotting.

#### IV. DOT- AND SLOT-BLOTTING

The dot- and slot-blotting procedures are essentially identical and can be used either as a qualitative method for rapidly screening numerous samples for the presence of antigen or antibody activity, or as a quantitative technique for determining antigen concentration. Quantitative versions are essentially similar to ELISA (see Chap. 29) and radioimmunoassays, except that the antigen is bound to a nitrocellulose membrane instead of plastic. Scanning densitometry is required for precise quantitation (see Sec. III.D) and there is little advantage over ELISA for this purpose, but as a qualitative or semiquantitative technique, dot- or slot-blotting has the advantages of speed and simplicity, with no sophisticated equipment necessary.

The procedure is as follows: Samples (0.5–5  $\mu\text{l}$ , usually as serial dilutions) are applied as a spot or rectangular slot to a strip or sheet of nitrocellulose and allowed to dry. The membrane is then blocked, overlaid with antibody, and developed as described earlier for immunoblotting. Larger sample volumes can be adsorbed to the membrane by using a vacuum manifold containing multiple filtration chambers with small holes or slots. Some models contain as many as 96 chambers, so that samples from a microtiter plate (e.g., hybridoma supernatants) can easily be assayed. A sheet of nitrocellulose is clamped under the wells, which seal against the membrane to prevent leakage between samples. The chambers are then filled with antigen solution, which is filtered through the membrane under vacuum, antigen being retained on the membrane. If different antibodies are to be tested, the whole blocking and overlay procedure can be performed in the apparatus. If the same antibody is to be used for each sample, the membrane is removed from the apparatus, blocked, and overlaid with antibody, and so on, in a dish.

In its simplest form as a rapid qualitative or semiquantitative assay, dot-blotting is useful for assessing parameters affecting the quality of immunoblotting (e.g., blocking conditions, optimal antibody dilution, washing buffers, and such). The sensitivity of a detection system can be determined by applying serial dilutions of antigen. An interesting variant is checkerboard immunoblotting (CBIB), in which antigens are applied to the membrane in narrow strips by means of a template with grooves cut into it. Antibodies are then applied, using the same template, but at right angles to the antigen strips. The result is a checkerboard pattern of reactivity in which 2000 or more antibody–antigen reactions can be performed within 24 h (Kazemi and Finkelstein, 1990a,b).

## V. ELISPOT ASSAYS

Membranes can also be used to measure the number of cells secreting specific antibody or other molecules—the ELISPOT assay. This has replaced the Jerne plaque-forming cell assay as a method of enumerating antibody-secreting cells, because of its greater reliability and versatility. In the original version, cells were cultured in standard plastic microwell plates that had been precoated with antigen. Antibody bound to the surface of the plastic at the bottom of the well was detected by overlay with substrate in agarose (Sedgwick and Holt, 1983), but this was improved on later by Möller and Borrebaeck (1985), who cultured lymphocytes on a nitrocellulose membrane sealed to the bottom of a 96-well microtiter plate (Millipore, UK). Antigen is bound to the membrane, and free binding sites are blocked with culture medium containing fetal calf serum (FCS). The plate is sterilized under a UV lamp for ½–1 h, and lymphocytes are cultured in each well at a suitable range of dilutions for anytime from 2 h to overnight at 37°C. The membranes are washed with PBS/0.05% Tween-20 by vacuum filtration, and antibody-secreting cells detected by overlay with enzyme-conjugated anti-immunoglobulin, followed by substrate. Single antibody-secreting cells appear as brown or blue spots.

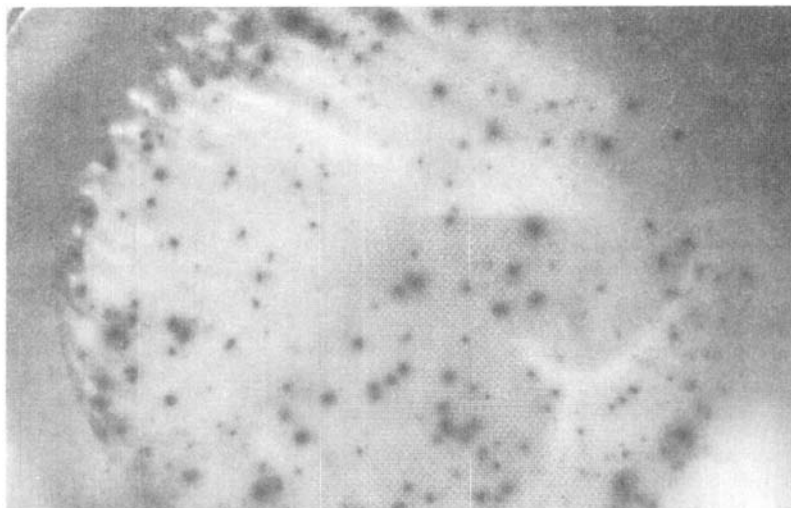
The principle of the assay is similar to immunoblotting. Antibody secreted by a cell resting on the membrane diffuses radially outward from the cell, and a proportion of the antibody molecules will bind to the antigen immobilized on the membrane. The cells are lysed by detergent during the washing steps, and the antibody–antigen complexes bound to the membrane can be detected as colored spots after incubation with anti-immunoglobulin and substrate.

It is possible to assay for two classes of antibody-secreting cells in the same well (e.g., IgG and IgM or IgA) by using HRP–anti-IgG and AP–anti-IgM or –IgA, incubating with both substrates, and counting the brown and blue spots (Czerkinsky et al., 1988), but in practice, we find it easier to perform the assays in separate wells. Barington et al. (1992) suggested that the cell culture and antibody overlay steps could be combined without loss of sensitivity. Klinman (1992) analyzed B cells secreting antibodies cross-reactive with DNA and TNP by sandwiching the cells between two plastic slides, one coated with each antigen.

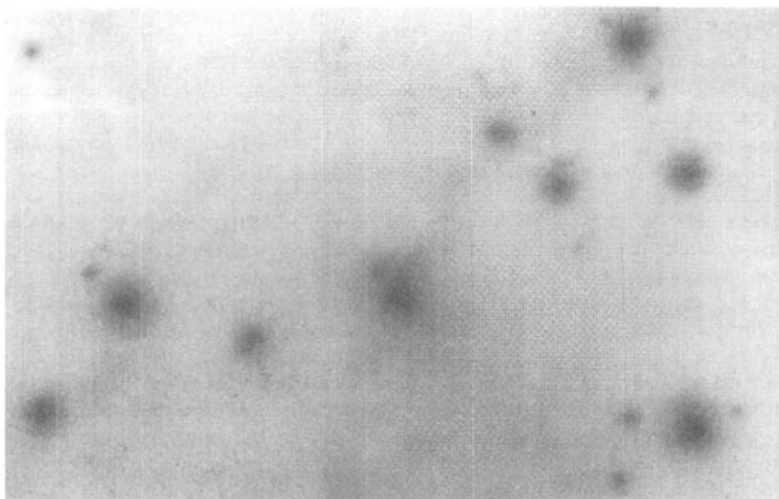
The assay can be reversed to enumerate cells secreting other molecules [e.g., it has been used to measure cells secreting the cytokines interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, interferon (IFN)- $\alpha$ , IFN- $\gamma$ , and tumor necrosis factor (TNF)- $\alpha$ ; Hutchings et al., 1989; Skidmore et al., 1989; Viselli and Mastro, 1989; Czerkinsky et al., 1991; Nordström and Ferrua, 1992; Fujihashi et al., 1993; Fig. 2]. Cytokine ELISPOT assays are normally performed using an antibody-capture system in which the membrane is coated with an anticytokine monoclonal antibody which “captures” cytokine molecules secreted by each cell. The cytokine–antibody complexes bound to the membrane are then detected by mono- or polyclonal antibody against different epitopes on the cytokine. If the second-layer antibody is of a different species from the first, the system can be amplified by a third-layer HRP- or AP-anti-immunoglobulin, or the biotin–avidin system can be used. Alternatively, the capture antibody can be omitted, allowing the cytokine to bind directly to the membrane (Viselli and Mastro, 1989; Czerkinsky et al., 1991).

## VI. APPLICATIONS OF IMMUNOBLOTTING AND DOT-BLOTTING

The power of immunoblotting and dot-blotting resides in the capacity of these techniques to resolve and identify components of complex mixtures in a large variety of systems. Immunoblotting has been successfully applied to the identification and characterization of many different microorganisms, both pathogenic and nonpathogenic, including bacteria, viruses, protozoa, yeasts, nematodes, and tapeworms. It has also been used to study the immune response and the



(a)



(b)

**Figure 2** ELISPOT assay for IFN- $\gamma$ -secreting cells photographed using a stereo zoom microscope. The membrane was precoated with monoclonal anti-IFN- $\gamma$  before adding concanavalin A-stimulated lymphocytes that were cultured on the membrane for 16 h. After washing off the cells, complexes of secreted IFN- $\gamma$  and anti-IFN- $\gamma$  antibodies bound to the membrane were detected with rabbit anti-IFN- $\gamma$  followed by HRP-goat antirabbit IgG. The photographs were taken at magnifications of (a)  $9\times$  and (b)  $32\times$ . (Courtesy of F.-P. Huang, Department of Immunology, University of Glasgow.)

specificity of antibodies against such organisms. An interesting adaptation of immunoblotting, with possible clinical applications, is the *immunoplot technique*, used to identify antigens of potential diagnostic value. An extract of an infectious organism is blotted against a large panel of sera from patients and healthy controls. The frequency of each band that stains with the panel of patients' sera is plotted against the appearance frequency of the same band among the control sera. Any antigens of diagnostic value (i.e., high frequency among the patients and low frequency among the controls) stand out above the rest (Tidey et al., 1993). The method has been applied to *Mycobacterium tuberculosis* (Espitia et al., 1989), *Entamoeba histolytica* (Ximenez et al., 1992), and tapeworms (Larralde et al., 1989). Several groups are working on the application of dot-blotting to produce cheap, simple dipstick assays for rapid diagnosis of infections in the field and the clinical laboratory without requiring sophisticated equipment.

Immunoblotting is also well suited to the study of allergens involved in type I (IgE-mediated) hypersensitivity reactions, which also have considerable clinical importance. They are often components of pollens, animal dander, house dust mite, yeasts, or fungi. They are difficult to purify, and the IgE antibodies responsible for allergic reactions, such as skin sensitivity, rhinorrhea, and asthma, are present at very low concentrations. Immunoblotting is very well suited to this type of problem and is being used to identify the active components of allergic reactions, to aid in their purification and structural analysis. Dot-blotting has potential applications for diagnosis of type I hypersensitivity reactions, in addition to being a very useful research tool.

Many of these applications have been reviewed previously (Stott, 1989). Some of the more exotic applications are described in the following.

### A. Affinity Purification of Antibodies

Small quantities of antibody (approximately 2–10  $\mu\text{g}$ ) can be affinity-purified by immunoblotting and subsequent elution of the bound antibody (Olmsted, 1988). This can be a viable alternative if pure antigen is not available for affinity chromatography, and monoclonal antibodies are unavailable. The method is as follows: The antigen extract is applied to the full width of a gel, electrophoresed, blotted, blocked, and overlaid with the antiserum in the normal way. A strip of nitrocellulose containing the antigen-antibody complex is then cut out and the antibody eluted with a low-pH buffer or chaotropic agent. Elution of antibody still bound to the antigen does not appear to be a problem. An interesting variation on this theme is to label the antibody with fluorescein isothiocyanate (or presumably any other label) while still bound to antigen on the membrane, followed by elution of the labeled antibody (Talian et al., 1983). This has the advantage of protecting the antigen-binding site during the reaction, since it is still occupied by antigen. Antibodies against plasmodial antigens have been purified by elution from recombinant expression proteins in lysed bacterial plaques bound to nitrocellulose. The eluted antibodies were used to detect native parasitic antigens by immunoblotting (Lyon and Weber, 1988).

### B. Antigens Recognized by T Lymphocytes

Lamb et al. (1988) have developed an immunoblotting assay, for antigens recognized by T cells, in which a mixture of antigens is separated by electrophoresis, blotted on to nitrocellulose, and the membrane cut into narrow strips at right angles to the direction of the tracks. The strips are sterilized and either added directly to the wells of a tissue culture plate or dissolved in dimethyl sulfoxide and precipitated as fine particles before addition to the plate. Peripheral blood lymphocytes or T-cell clones are cultured with the blotted proteins, and cell proliferation is assayed by incorporation of [ $^3\text{H}$ ]thymidine. The profile of antigens recognized by the T cells can be determined from their ability to induce a T-cell response.



The technique has been used to analyze T-cell antigens of influenza virus, mycobacteria, and house dust mite (Young and Lamb, 1986; Lamb et al., 1987; Lamb and Young, 1987; O'Hehir et al., 1987). It is not clear how the antigens are presented in this system, but they are probably processed by peripheral blood mononuclear feeder cells and presented as peptides bound to major histocompatibility complex (MHC) class I or class II antigens.

T-cell antigens can also be analyzed by 2-D PAGE. Gulle et al. (1990) used an adapted semidry blotting apparatus to elute mycobacterial antigens from a 2-D gel into 480 wells cut in a master plate, the antigens being maintained in the soluble phase throughout. Each fraction was analyzed for its ability to stimulate T-cell proliferation *in vitro*, producing a two-dimensional pattern of reactivity.

### C. Autoantigens and Autoantibodies

Immunoblotting is a powerful technique for investigations of the autoimmune response in autoimmune diseases and animal models of autoimmune diseases; for example, it has been used to analyze the complex nuclear protein antigens and ribonuclear protein (RNP) particles to which autoantibodies are found in systemic lupus erythematosus, mixed connective tissue disease, and Sjogren's syndrome; mitochondrial antigens in primary biliary cirrhosis; thyroid antigens in autoimmune thyroiditis; and many other autoimmune diseases have yielded to this approach. Before the development of immunoblotting, such antigens could be studied only by conventional immunochemical techniques, such as immunodiffusion or fluorescence microscopy, which did not allow separation of the different antigenic and nonantigenic components often present in association with each other.

The specificity, class, subclass, and idiotype of autoantibodies can also be identified (e.g., idiotypes of rheumatoid factors, anti-DNA antibodies, and cryoglobulins, all have been identified by immunoblotting). Monoclonal IgG rheumatoid factors were identified by their ability to bind SDS-denatured human Fc fragments separated from contaminating IgG by SDS-PAGE and blotted on to nitrocellulose (Newkirk, 1992). The rheumatoid factor antibodies were detected using F(ab)<sub>2</sub> antihuman light chain.

### D. Epitope Mapping

The epitopes of protein antigens can be mapped by partial digestion, immunoblotting the resulting peptides, and probing with monoclonal antibodies. The phosphorylation sites on a multifunctional protein (CAD) have been mapped by blotting polypeptides generated by limiting proteolysis and direct digestion of the bands on nitrocellulose with trypsin. Similarly, Luzio and Jackson (1988) defined epitopes on complement component C9, concerned with formation of the membrane attack complex, by chemical and enzymic cleavage, combined with immunoblotting. Some of the other proteins for which epitopes have been mapped in this way are fibronectin,  $\alpha_2$ -macroglobulin, herpes simplex virus, Newcastle disease virus, histone H1, myelin basic protein (Sheng et al., 1988) and cholera enterotoxin (Kazemi and Finkelstein, 1990b).

### E. Immunization with Blotted Proteins

An individual band identified by immunoblotting can be cut out of the membrane and used to raise a specific antiserum or monoclonal antibody. The band can be chopped finely and injected subcutaneously or intraperitoneally; alternatively, it can be eluted with a nonionic detergent, or the nitrocellulose can be dissolved in acetone, methanol, or dimethyl sulfoxide and injected. With this approach, antibodies have been produced by intrasplenic immunization of mice with as

little as 70 ng of protein blotted on to a membrane (Larsson and Nilsson, 1988; Forrest and Ross, 1993). Larger amounts ( $\geq 0.8 \mu\text{g}$ ) stimulated an immune response after intraperitoneal injection. Antigen, blotted on to a PVDF membrane, has been used to make mouse monoclonal antibodies by *in vitro* immunization (Guzman et al., 1993). Strips of membrane containing the antigen were cut into small pieces and cultured with unimmunized mouse spleen cells in conditioned medium, containing muramyl dipeptide.

This could be envisaged as a "boot-strap" method of purifying an antigen, since the blotted material can be used to produce an antibody which, in turn, could then be used to affinity-purify the same antigen in larger amounts.

## F. Spectrotype Analysis of Antibodies by Reverse Immunoblotting

Isoelectric focusing (IEF) in thin-layer gels separates molecules by focusing them into narrow bands at their isoelectric points in a pH gradient. It differs from electrophoresis in that the molecules move along the gradient until they reach the position at which the pH of the medium is equal to their pI, at which point their net charge is zero and, therefore, they reach equilibrium. The technique has been used to analyze complex mixtures of proteins and has much higher resolving power than electrophoresis, being capable of distinguishing large molecules differing by only a single, charged amino acid. The most extensive use of IEF, however, has been for analysis of antibody spectrotypes. Antibody secreted by a single clone of plasma cells focuses into a unique pattern of closely spaced bands—the clonotype (owing to microheterogeneity). The spectrotype of antibody in a polyclonal response is the pattern of bands on an IEF gel, consisting of the sum of the clonotypes of all the responding clones of plasma cells. Thus, it is possible to analyze the number of responding B-cell clones and follow changes in the behavior of individual clones during an immune response by observing the IEF spectrotype of the antibodies. For antibodies against low- $M_r$  molecules (haptens), the spectrotype can be developed by simply overlaying the gel with radioactive hapten, but high- $M_r$  antigens or antibodies penetrate polyacrylamide gels, commonly used for IEF, so slowly that the method becomes impractical.

Isoelectric focusing in agarose gel facilitates penetration by macromolecules, owing to the larger pore size of agarose. Overlaying with anti-immunoglobulin, followed by staining of the precipitated proteins, has been used to identify paraproteins in patients' sera and urine (Sinclair et al., 1986a) and to quantitate monoclonal immunoglobulins present in serum (Sinclair et al., 1986b). The greater sensitivity of immunoblotting has been exploited for the detection of paraproteins in patients with B-cell neoplasia and to study oligoclonal-banding patterns in cerebrospinal fluid (Nespolo et al., 1987).

Specific antibodies against high- $M_r$  antigens can also be spectrotyped by focusing, blotting, and overlay with labeled antigen. We have coined the phrase *reverse immunoblotting* for this technique, since here, it is the antibodies that are separated on the gel, and the antigen is used as the probe (Stott and McLearie, 1986). We have used this approach to investigate qualitative and quantitative changes in autoantibody spectrotypes and in the behavior of B-cell clones secreting autoantibodies against thyroglobulin, ssDNA, dsDNA, and retinal S-antigen in human patients and animal models of autoimmune thyroiditis (Stott et al., 1986; 1988a), systemic lupus erythematosus (Stott et al., 1986; 1988b; 1990; Stott, 1992), and allergic uveoretinitis (Forrester et al., 1989a,b). Grimaldi et al. (1988) used a similar method to identify antibodies of restricted heterogeneity against gp120 and p24 human immunodeficiency virus (HIV) antigens in acquired immunodeficiency syndrome (AIDS) patients.

Focusing in polyacrylamide restricts the spectrotype to IgG antibodies, whereas IgA and

IgM will focus in agarose gels, although better spectrotypes result if the latter are subjected to mild reduction to dissociate them into subunits, especially IgM, which tends to precipitate out and smear in the gel owing to its insolubility at low ionic strength.

An alternative approach is to blot the antibodies on to nitrocellulose sheets precoated with antigen (affinity immunoblotting). Additional information about the class, subclass, or allotype of the antibodies can be obtained by use of appropriate anti-immunoglobulin to detect the bound antibodies. Monoclonal immunoglobulins in serum and other body fluids can be identified by blotting on to nitrocellulose coated with anti-immunoglobulin. McLachlan (1989) used this method to identify low concentrations of monoclonal immunoglobulin in serum, and Kaiser (1991) observed oligoclonal-banding patterns of IgG, IgA, and IgM in cerebrospinal fluid. Jauberteau et al. (1990) used affinity blotting to detect serum monoclonal IgM antibody against glycosphingolipids.

### G. Microsequencing

Proteins blotted on to a membrane can be sequenced directly without further purification, provided they are sufficiently well separated. Choli et al. (1989) and Pappin et al. (1990) successfully sequenced proteins immobilized on PVDF membranes. The latter trapped the protein on the membrane by coating with polyalkylamine, which was then crosslinked to the blotted protein, enabling sequencing to be performed in the solid-phase, with the protein still bound to the membrane. Petersen et al. (1992) sequenced timothy pollen allergens after blotting from a 2-D PAGE gel.

## VII. SUMMARY AND FUTURE DEVELOPMENTS

The development of the immunoblotting technique, and the associated technique of dot-blotting, arose from the need to combine the resolving power of electrophoresis, either in one or two dimensions, or IEF with the specificity of antibodies as probes for identification of molecules present in complex mixtures, or even regions within a single large molecule, such as a protein. With the realization of the limitations of earlier methods, in which radiolabeled proteins were immunoprecipitated and separated by SDS-PAGE, the development of immunoblotting resulted in an explosion of publications describing its application to many different problems, only a few of which have been described here. These include studies on molecules produced by pathogenic and nonpathogenic microorganisms; not only their surface antigens, but also internal molecules essential to their metabolism. The results of this work are already providing a better understanding of the nature and interrelations of microorganisms and the immune response against them. The simplicity and speed of dot-blotting, combined with the reliability and precision of monoclonal antibodies, has great potential as a simple, rapid test for use in the clinical laboratory. A few of the research applications have been described here, and we are likely to see many new developments in the future. Technical developments may include better membranes, including substrates that bind molecules covalently and that form reversible covalent bonds so that antigens can be eluted after blotting for purification and structural studies. The speed and sensitivity of detection systems, such as chemiluminescence, are also likely to undergo further development and new applications.

The ELISPOT assay also has great potential. The reason it has not become more widely used is probably because of the tediousness of counting large numbers of spots visually, but the now greatly improved (though expensive) image analysis equipment should make it possible to automate this procedure. Assays for cells secreting other cytokines, hormones, and other molecules are likely to be developed in the near future.

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