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Review Article

Immunoblotting and Dot Immunobinding — Current Status and Outlook

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General Introduction

In recent years, solid-phase immunoassay techniques have found wide application in basic research and clinical applications. The high sensitivity which can be obtained with labelled ligands, and the simple separation of bound from unbound reagents make this type of immunoassay particularly attractive. However, the methodology on its own yields no information on the nature of the reacting antigen. To determine homogeneity or identity of unlabelled antigens the classical methodologies of immunodiffusion and immunoelectrophoresis relying on precipitation are available. More recently, as an extension of gel overlay techniques, the development of methods for transferring proteins from the gel phase of electrophoresis to a solid phase for immunoreaction permitted the optimal combination of high resolution gel electrophoresis with the sensitivity and simplicity of solid-phase assays.

The first practical methods for transfer of proteins from gel electropherograms to a suitable solid phase were published in 1979. Renart et al. (1979) introduced transfer by diffusion to activated cellulose to provide covalent binding, and we (Towbin et al., 1979) described the transfer in an electric field and non-covalent binding to nitrocellulose. Diffusional transfer to nitrocellulose was reported by Bowen et al. (1980). In the intervening years, there has been an explosive increase in applications of these techniques, as well as numerous attempts to improve on the original protocols. We will attempt to review the various strategies and also summarize some applications (for an earlier review on this subject cf. Gershoni and Palade, 1983).

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The distinctive feature of this methodology is the ability to retain the original pattern of the proteins on a solid phase during the course of immunoassay procedures. This feature can also be used in immunoassay where the resolving power of electrophoresis is not needed. Thus, antigens can be applied in the format of dots or spots (Glenney et al., 1982; Hawkes et al., 1982; Herbrink et al., 1982; Huet et al., 1982; Yen and Webster, 1982). This permits the use of any desired geometry, and has the advantages of permitting multiple simultaneous assays, high sensitivity, and simple operation (Hawkes et al., 1982; Gordon et al., 1983).

There has also been a proliferation of terminology for these methodologies. We prefer the terms used in the title as they are descriptive of the actual processes. Blotting implies some flow transfer from one phase to another, and dot implies direct application in the form of a dot. Other widely used terms are 'Western blotting' and 'protein blotting'. Dot immunobinding has also variously been referred to as spot immunodetection (Huet et al., 1982), dot blot immunoassay (Yen and Webster, 1982) or antigen spot detection (Herbrink et al., 1982).

Technical Features

Immunoblotting: transfer

Various principles have been utilized to transfer proteins to membranes (see Tables I and II). Diffusion blotting can be performed without special equipment. There are no restrictions in the use of buffers which might be considered useful to induce renaturation or adsorption of antigens. Some resolution may be lost during the lengthy diffusion times required for transfer. Peferoen et al. (1982) invented a

TABLE I
EXAMPLES OF TRANSFER BY DIFFUSION AND SOLVENT FLOW

Renart et al. (1979)	To DBM paper from SDS gels after equilibration with phosphate buffer and periodate cleavage, 16 h
Schaltmann and Pongs (1980)	To cellulose acetate from SDS gels in phosphate-buffered saline, 18–24 h
Bowen et al. (1980)	To NC ^a from SDS gels after equilibration in Tris buffer, pH 7, containing 4 M urea, 36–48 h
Reinhart and Malamud (1982)	To NC from thin, non-denaturing IEF gels in 10 mM Tris-HCl, pH 8, 2 h
Lanzillo et al. (1983)	To NC from agarose IEF gels, 3 h
Aubertin et al. (1983)	To NC directly from SDS gels in Tris buffer, pH 7, containing 4 M urea and 0.1% SDS, overnight
Peferoen et al. (1982)	To NC from SDS and IEF gels by vacuum induced solvent flow, 20–60 min

^a NC: nitrocellulose membrane.

TABLE II
EXAMPLES OF ELECTROPHORETIC TRANSFER

Towbin et al. (1979)	To NC ^a from urea gels, in 0.7% acetic acid, 1 h at 6 V/cm; to NC from SDS gels, in 25 mM Tris-192 mM glycine-20% methanol, 1 h at 6 V/cm
Bittner et al. (1980)	To DBM paper from SDS gels, in 25 mM phosphate buffer, pH 6.5, ca. 10 V/cm, 1 h
Reiser and Wardale (1981)	To diazophenylthioether paper from SDS gels (prepared in borate/sulphate buffer) in 10 mM sodium borate, pH 9.2, 1 h at 32 V/cm
Erickson et al. (1982)	To NC from SDS gels in 25 mM Tris-192 mM glycine (pH 8.3)-20% methanol-0.1% SDS, 60 Vh/cm
Nielsen et al. (1982)	To NC from SDS gels in 25 mM Tris-192 mM glycine-0.01% SDS, 15 min at 6 V/cm; or in the same buffer containing 20% methanol and 0.01–0.02% SDS, 1 h at 6 V/cm
Gershoni and Palade (1982a)	To ZB ^b from SDS gels, in 15.6 mM Tris-120 mM glycine, pH 8.3, with or without 20% methanol, 4–5 V/cm, 2 h
Sutton et al. (1982)	To NC from non-denaturing gradient gels and IEF gels, in 0.7% acetic acid, from SDS gels, in 10 mM Tris-77 mM glycine-0.1% SDS, pH 8.3, 60–90 min at 15 V/cm

^a NC: nitrocellulose membrane.

^b ZB: Zeta-bind.

fast blotting procedure where a liquid flow across the gel is induced in a vacuum drier. Similar to diffusion blotting, this set up allows free choice of transfer buffer. Electrophoretic transfer is fast and can be quantitative. The original procedure described (Towbin et al., 1979) was designed to obtain a faithful replica of the gel in the shortest time possible and requiring the least handling. This goal was achieved with ribosomal proteins separated in polyacrylamide gels containing urea. Elution from SDS polyacrylamide gels posed more problems. In the presence of SDS proteins poorly adsorb to nitrocellulose. The rationale behind electrophoretic elution from SDS gels was that proteins are still complexed with SDS and retain their negative charge. The proteins then move towards the anode and adsorb to the membrane in an environment without SDS. Methanol was included in the buffer to avoid swelling of the gel during transfer and was also found to improve adsorption of proteins. Addition of low concentrations SDS to the transfer buffer improved the transfer of a variety of proteins (cf. Table II). Modification of the electrolytes in the transfer buffer has little influence on transfer yields (Gershoni and Palade, 1982a). The use of buffers of lower ionic strength such as described by Sutton et al. (1982) should allow higher voltages to be applied.

Small proteins have been reported to move through nitrocellulose membranes at high current density (Lin and Kasamatsu, 1983). When membranes of smaller pore size (0.1 μ m) were used loss of these proteins was reduced. It seems that a universally applicable, optimal transfer buffer compatible with complete absorption to the

membrane has not yet been formulated and that transfer conditions should be optimized for best results.

To facilitate elution of large proteins the gel can be made more porous by breaking the gel matrix chemically (Renart et al., 1979). This procedure may be too harsh for many proteins (Reiser and Wardale, 1981). Swelling the gel prior to transfer in aqueous transfer buffer or transfer buffer containing urea may also speed up elution by increasing the pore size. A different approach was taken by Gibson (1981) who partially digested the proteins in situ before transfer.

Elution following proteolytic digestion is also the basis of a sensitive assay for electrophoretically separated proteases (Miskin and Soreq, 1981). In this assay radiolabelled peptides released from a non-diffusible substrate incorporated into the gel are trapped on a sheet of nitrocellulose.

The findings that SDS improves elution by diffusional (Aubertin et al., 1983) and electrophoretic transfer (e.g., Erickson et al., 1982; Gershoni and Palade, 1982a; Nielsen et al., 1982; Sutton et al., 1982) suggests that poor elution is due to precipitation within the gel.

Chemically activated papers yield a covalent bond between protein and matrix and may have the advantage that detergents can be used subsequently without fear of losing protein. Although some detergents, e.g., Nonidet P-40, may remove protein from nitrocellulose (Lin and Kasamatsu, 1983) the absorptive membranes have become widely accepted. Some of their attractive features are easy handling, high binding capacity and homogeneity. The forces retaining proteins so efficiently on nitrocellulose are poorly understood. 'Hydrophobic' forces very likely play an important role. Since hydrophobic forces increase with ionic strength 0.2 M magnesium chloride was used as part of a quantitative protein assay to adsorb proteins to nitrocellulose (Kuno and Kihara, 1967), where they are subsequently stained.

Proteins transferred to nitrocellulose can also be covalently fixed with glutaraldehyde (Kay et al., 1983) or the heterobifunctional crosslinking reagent *N*-hydroxysuccinimidyl-*p*-azidobenzoate (Kakita et al., 1982).

Zeta-bind is an inert matrix substituted with quarternary ammonium groups. It has very high binding capacity (Gershoni and Palade, 1982a), and consequently larger amounts of protein may be loaded on the gel to detect minor antigens. The high binding capacity of Zeta-bind requires extensive blocking to prevent non-specific binding. Ideally, the binding capacity of the membrane should be matched to the protein loaded on the separating gel since a high excess binding capacity also brings along the danger of non-specific absorption of the detection reagents.

Staining of total protein patterns on blots

General protein stains are amido black, fast green and the recently described Indian ink stain (Hancock and Tsang, 1983). The silver 'staining' technique of Yuen et al. (1982) only works for SDS gels and the background is stained, not the proteins. These staining procedures, except for the last one, may impair antigenicity of the blotted proteins and diminish visibility of the colour deposited in enzyme-linked detection systems. Especially concerning proteins separated in 2 dimensions, one should be able to align precisely the total protein pattern with the immunostained

TABLE III
EXAMPLES OF BLOCKING CONDITIONS

Membrane	Antigen	Blocking reagent	Comments	References
NC	Ribosomal proteins	5% BSA, 1 h, 40°C 10% horse serum, 1 h, 40°C		Towbin et al. (1979) Gordon et al. (1982)
NC	Bacterial membrane proteins	0.05% Tween 20, 1 h, 37°C	Protein staining after immune detection possible	Batteiger et al. (1982)
NC	Synaptosomal membrane proteins	0.05% Tween 20, 5 min, 22°C		DeBlas and Cherwinski (1983)
ZB	Various	10% BSA or 1% haemoglobin, 45°C, overnight		Gershoni and Palade (1982)
DBM	Viral proteins	0.1 M Tris-10% ethanolamine, 0.25% gelatine, pH 9, 2 h, 37°C	Chemical inactivation and protein blocking	Renart et al. (1979)

antigen. For this purpose a reversible staining method has been devised (Towbin et al., 1982) in which the nitrocellulose-bound proteins are sequentially incubated with heparin and toluidine blue at low pH. The pattern appearing can then be recorded and erased by incubation with a buffer of high ionic strength containing methanol *. Batteiger et al. (1982) used 0.05% Tween 20 in all buffers and blocking solutions. This allowed the nitrocellulose to be stained with amido black after immune detection. Transferred proteins can also be derivatized with a hapten and the tagged proteins can later be visualized with anti-hapten antibodies (Wojtkowiak et al., 1983). Of course, radiolabelled molecular weight markers or other suitable protein mixtures may be used to obtain a reference pattern.

Blocking of immunoblots

Before proceeding with the immune reaction one has to prevent non-specific binding of the reagents to the membrane. Adsorbing membranes (nitrocellulose and Zeta-bind) must be blocked. Chemically activated membranes must be inactivated to saturate unoccupied binding sites (examples of blocking conditions are given in Table III). There are 2 aspects to the blocking step: it is essential to reduce

* An unpublished milder method for reversible protein staining is as follows: sequentially incubate the blot with 6 M urea, phenol red (50 mg/l) in 1 mM HCl, briefly wash with 1 mM HCl. Proteins stain yellow and for better contrast may be viewed through a blue filter. Alternatively, the membrane may be blotted dry and exposed to ammonia vapour and proteins appear red. The dye is washed out in buffered saline and the blot can now be processed as usual.

non-specific binding and it may promote renaturation of antigenic sites. With regard to renaturing, Petit et al. (1982) observed that treatment of antibodies blotted from SDS gels with Nonidet P-40 accelerated reappearance of idiotypic determinants. Similarly, incubation of blotted synaptosomal proteins with 8 M urea appeared to increase their subsequent reactivity (Erickson et al., 1982). Even simply extending the blocking procedure from the usual 1 h to 2 h (at 40°C) improved the signal in the case of ribosomal proteins (Towbin, unpublished). It is likely that many monoclonal antibodies which fail to react with protein antigens blotted from SDS gels would do so if better renaturing conditions were available. Alternatively, electrophoretic conditions less denaturing to the antigen could be chosen. In this respect the considerations given to the problem of renaturing receptors (discussed below) should also apply to renaturation of antigenic sites.

For nitrocellulose, gelatine alone as a cheap and immunologically inert blocking agent is not satisfactory. We routinely use 10% horse serum for most applications. Others (Burnette, 1981; Peferoen et al., 1982) also stressed the superior blocking properties of whole serum. Where antibody reactions with serum components are conceivable, BSA may be used. Again, mild detergents (Tween 20) as blocking agent and buffer component may be used in many instances (Batteiger et al., 1982). Especially for detection of low amounts of antigen, proper choice of blocking conditions are important.

Immunodetection on blots

Incubation times and antibody dilutions vary in a wide range depending on the antibody and amount of antigen. For antibodies of high avidity high dilutions and long incubation times may be used in order to save precious sera as is the case for anti-hormone sera. For low avidity antibodies (with half-life up to 3 h) it is better to use a high antibody concentration for a short time (due to the fast dissociation longer incubation is useless) and also to use the secondary antibodies concentrated and for a short time.

Calculation from literature data as well as our own experience with purified high affinity monoclonal antibodies indicates that antibody concentrations of about 10^{-9} M (= 0.15 µg/ml for IgGs) applied for about 1 h are usually sufficient. It is also possible that the primary antibody binding to antigen is stabilized by the secondary labelled antibody in an immunofixation process.

In the case of detection with protein A, whole serum may not be used in blocking solutions. The binding of protein A varies between species and isotypes; rabbit antibodies are known to bind best to protein A and hence are recommended as bridging antibodies in the detection of non-binding isotypes. Amplification will result from the binding of several antibodies to the primary bound antibody. If the primary bound antibody is monoclonal, the bridging antibody will permit the binding of the tertiary labelled antibody which is no longer restricted by the isotype of the monoclonal.

Peroxidase is the most widely used enzyme label. Table IV shows that virtually all combinations of amplifying reagents developed for immunohistochemistry have been tried on blotted proteins. Incidentally, nitrocellulose-bound proteins have been

suggested as ideal 'histological specimens' on which staining techniques can be standardized (Larsson, 1981; Bosman et al., 1983). Due to the variability of the quality of immune reagents comparison of different staining procedures is difficult. In order to test enzyme conjugates we dot serial dilutions of antibody or myeloma proteins of different isotypes and test the conjugates at different dilutions. The detection limit with anti-immunoglobulin reagents coupled to peroxidase is about 0.1 ng Ig (applied as a 0.5 μ l spot on nitrocellulose (Hawkes et al., 1982) or 1 fmol. With radioactive antibodies labelled to a high specific activity this limit may be reduced between 10- and 100-fold. Whether this sensitivity can also be achieved with blotted proteins depends on the degree of antigen renaturation, suppression of background staining as well as sufficient concentration, affinity and incubation times of the immune reagents.

Sample application for dot immunobinding

Several precursors to this method have been described. Larsson (1981) devised a multi-spot test for peptide antigens on paper where the antigens were fixed with formaldehyde vapour. Cleveland et al. (1979) and Richmann et al. (1981) immobilized herpes-virus-infected cells to paper with a filtration manifold, followed by immunodetection. By analogy with the blotting method of Renart et al. (1979), Herbrink et al. (1982) used activated cellulose as a support for the antigens.

The visibility of a dot depends on the contrast of the colour generated against the background and is thus dependent on the density and not the amount of antigen in a dot. Hence, it is more economical to apply antigens in small volumes of concentrated antigen. Volumes of 0.5 or 1 μ l can be delivered with repetitive microdispensers. More recently, we have used a programmable syringe drive device (Hamilton Microlab M) to deliver repetitively down to 0.1 μ l volumes (Derer et al., 1984; Gordon and Rosenthal, 1984b). Nitrocellulose with grid printed on it permits easy application of the dots to a defined location and the subsequent cutting of strips of desired geometry.

Dry nitrocellulose easily soaks up volumes of the above size range, and binds the material to a tight dot. For larger volumes the use of multiple filtration manifolds has been described (Holmberg et al., 1983), where the sample is applied under vacuum, and subsequent incubations are performed in the wells of the filtration apparatus and not on the entire sheet. The specific adsorption capacity of nitrocellulose is so high that Holmberg et al. (1983) were able to apply hybridoma culture supernatants with no purification or concentration. Bennet and Yeoman (1983) used a manifold constructed from a microtitre plate to maintain separate individual reactions on the same sheet. In our hands such methods cause difficulties due to seepage between wells through the nitrocellulose (Sun and Gordon, unpublished).

For antigens insoluble in aqueous medium, it is not necessary to use nitrocellulose. Thus, Esen et al. (1983) applied an ethanol solution of zein to paper. We have found 50% ethanol is optimal for applying cardiolipin to nitrocellulose (Sun and Gordon, in preparation).

When highly diluted antigens are applied, it is important to use a carrier for the sample solution. We routinely use bovine serum albumin (BSA) for this purpose.

TABLE IV
EXAMPLES OF IMMUNO-DETECTION PROCEDURES

Antigen (minimum amount detected)	Detection procedure	References
<i>Detection by radioactivity</i>		
Ribosomal proteins	1. Mouse antiserum, 2. ^{125} I-sheep anti-mouse IgG	Towbin et al. (1979)
Viral proteins (1 ng)	1. Antiserum, 2. ^{125} I-protein A	Renart et al. (1979); Burnette (1981)
Intermediate filaments	1. moAb ^a , 2. ^{125} I-F(ab') ₂ rabbit anti-mouse Ig	Pruss et al. (1981)
Hepatitis B surface antigen (1 pg)	1. ^{125} I-antibody	McMichael et al. (1981)
Epstein-Barr virus and rheumatoid arthritis nuclear antigen	1. Human positive serum, 2. human negative serum (complement source), 3. ^{125} I-anti-C4 (a complement fixation assay)	Billings et al. (1983)
<i>Detection by peroxidase (PO) conjugates</i>		
Ribosomal protein (0.1 ng)	1. Goat antiserum, 2. rabbit anti-goat IgG/PO, 3. <i>o</i> -dianisidine/H ₂ O ₂	Towbin et al. (1979)
Human IFN- β	1. moAb, 2. rabbit anti-mouse IgG/PO, 3. goat anti-rabbit IgG/PO, 4. <i>o</i> -dianisidine/H ₂ O ₂	Hochkeppel et al. (1981)
Dehistonized chromatin	1. Rabbit antiserum, 2. anti-rabbit IgG, 3. PAP ^b , 4. DAB/H ₂ O ₂ ^c	Glass II et al. (1981)

Various antigens (0.1 ng antibody)	1. mono- or polyclonal antibody, 2. antibody/PO, 3. 4-chloronaphthol/H ₂ O ₂ (dot immunobinding assay)	Hawkes et al. (1982)
Synaptosomal plasma membranes	1. moAb, 2. rabbit anti-mouse Ig, 3. PAP, 4. DAB/H ₂ O ₂ in presence of nickel and cobalt ions with osmium postfixation	DeBlas and Cherwinski (1983)
Angiotensin-1-converting enzyme (150 ng)	1. Rabbit antiserum, 2. biotinylated goat anti-rabbit Ig, 3. avidin-biotinylated PO complex, 4. 3-amino-9-ethylcarbazol/H ₂ O ₂	Lanzillo et al. (1983)
Pituitary growth hormone (1 pg, spotted on NC)	1. Rabbit serum, 2. sheep anti-rabbit IgG, 3. PAP, 4. repeat steps 2 and 3, 5. DAB/H ₂ O ₂	Meuris et al. (1983)
<i>Toxoplasma</i> proteins	1. Patient's serum, 2. class-specific rabbit anti-human IgG or IgM/PO, 3. DAB/H ₂ O ₂	Partanen et al. (1983)
<i>Detection by alkaline phosphatase (AP) conjugates</i>		
<i>Salmonella</i> cytoplasmic proteins	1. moAb, 2. F(ab') ₂ rabbit anti-mouse F(ab') ₂ /AP, 3. naphthol ASMX phosphate/fast red TR salt	O'Connor and Ashman (1982)
Human IgG	1. Anti-IgG/AP, 2. α -naphthyl phosphate/fast blue B	Jalkanen and Jalkanen (1983)
Various proteins	1. moAb, 2. sheep anti-mouse Ig/AP, 3. β -naphthyl-phosphate/fast blue B	Turner (1983)

^a moAb: monoclonal antibody.

^b PAP: peroxidase anti-peroxidase complex.

^c DAB: diaminobenzidine.

Occasionally antibodies bind to BSA. This can be avoided by including BSA together with horse serum in blocking solution and antibody diluents (Gordon and Kuster, unpublished results).

Dot immunobinding: blocking

The remainder of the dot immunobinding protocol corresponds exactly to that of immunoblotting. As with immunoblotting, it has been our experience that total horse serum provides the most versatile, straightforward blocking agent. It is useful for screening both monoclonal antibodies and human pathological sera. Foetal calf serum is both less effective and more expensive, and should therefore not be used.

When assaying nucleic acids as antigens the presence of nucleases in whole serum is a problem. While serum can be used with anti-DNA antibodies (Rordorf et al., 1983; Gordon and Rosenthal, 1984a,b), degradation can occur with some other polynucleotides (Saarma and Gordon, unpublished results). For screening specificities of hybridomas from autoimmune mice, we have found that 1 mg/ml BSA and 0.1% Nonidet P-40 is optimal for blocking (Sun, Kuster and Gordon, in preparation).

Immunodetection on dots

For detection of antibodies bound to the dot, immunoperoxidase staining is, in our hands, extremely sensitive and most convenient. Others (Herbrink et al., 1982; Huet et al., 1982; Yen and Webster, 1982) used ^{125}I -labelled protein A detection. Bosman et al. (1983) found similar sensitivity with peroxidase-anti-peroxidase detection compared with peroxidase-coupled immunoglobulin. Holmberg et al. (1983) used biosynthetically labelled immunoglobulin in culture supernatants to detect anti-immunoglobulin bound to nitrocellulose.

Detection of analytes with antibodies bound to the solid phase is also possible within the dot immunobinding assay format. Thus, we (Towbin et al., 1982) and McDougal et al. (1983) have devised a sandwich assay for immunoglobulin typing in culture supernatants, and we (Derrer et al., 1984) have devised sandwich assays for total IgE determination.

Re-use of blots and recovery of antibodies

Nitrocellulose, DBM paper or Zeta-bind with bound proteins may be repeatedly probed with different antisera or other ligands (Renart et al., 1979; Legocki and Verma, 1981; Gershoni and Palade, 1982a), thus saving protein and time. In addition to the desorption procedures known from affinity chromatography (choatropic ions, low pH, competing ligands) harsher treatments of the blots (SDS (Symington et al., 1981), or urea (Erickson et al., 1982)) have been used to remove all ligands.

Antibodies eluted from 1 band in a blot are affinity purified reagents (Olmsted, 1981) and can be used for immunofluorescence studies or for further immunochemical characterization. As an example antibodies from an antiserum against vinculin being affinity purified by this method were shown to cross-react with a 150 kD protein on blots (D'Angelo Siciliano and Craig, 1982).

Quantitation

While immunoblotting is a powerful qualitative procedure, it can also be used as a sensitive, quantitative solid-phase immunoassay. In the case where the analyte is bound to solid phase a prerequisite is complete or at least reproducible absorption of the protein mixture to the solid phase. In contrast to absorption to plastic plates which only have a low binding capacity, this condition is nearly ideally fulfilled by nitrocellulose membranes.

Another advantage of quantitative use of blots is that other cross-reacting or interfering substances are likely to be separated out in the electrophoresis step. Accordingly, Howe and Hershey (1981) established a quantitative analysis for bacterial initiation factors by running bacterial lysates on SDS gels and probing the blots with ^{125}I -labelled protein A. The results showed a linear relation between amount of antigen and sensitivity down to 1 ng. Vaessen et al. (1981) used a similar method to quantitate yeast cytochrome bc_1 complex. Batteiger et al. (1982) measured bound rabbit antibodies with iodinated protein A.

We also used densitometric quantitation of the immunoperoxidase stain on immunoblots with reflectance densitometry equipment (Towbin et al., 1982; Rordorf et al., 1983). Guengerich et al. (1982) also used densitometry to quantitate immunoperoxidase stained cytochrome P-450 in blots. We applied the same methodology for dot immunobinding (Hawkes et al., 1982) and showed a quantitative relationship between amount of immunoglobulin on the nitrocellulose and densitometric response. Calibration curves with known amounts of dotted immunoglobulin were set up and a modified logarithmic relationship was found to fit the data over a wide range ($y = A \log(x + B)$ where y is the densitometric absorbancy, x is the amount of immunoglobulin bound and A and B are empirical constants (Gordon and Rosenthal, 1984b). An even better fit is observed with a relationship of the form: $1/y = A/x + B/x^2 + C$. We have used the same methodology for sandwich type assays for IgE (Derer et al., 1984). This assay could be quantitated down to ca. 100 pg/ml. A further dot assay system has been developed for the interferon induced oligo-nucleotide 2'5'-oligo-adenylate (Gordon and Minks, 1981). We have prepared specific antibodies against this oligonucleotide and showed that in a competition assay with 2'5'-oligo-adenylate coupled to bovine serum albumin in the dot, we can achieve sensitivities in the pmol range (Minks and Gordon, in preparation).

For automatic densitometric quantitation of dot immunobinding data we have written a computer program to sort out the signals generated by the grid lines and to display the data in a histogram format (Fig. 1).

Epitope mapping

Fragmentation of proteins and immunological analysis of the peptides on blots (termed epitope mapping (Clark, 1983)) have served to characterize binding sites on proteins and to probe the relationship between cross-reactive proteins. Thus, Reiser and Wardale (1981) studied SV40-induced proteins by 2-dimensional electrophoresis with intermediate proteolytic digestion. Monoclonal antibodies were used in mapping studies of fibronectin (Vartio et al., 1982) and of spectrin (Yurchenco et al.,

1982.) Mendel-Hartvig and Nelson (1983) studied the topology of a membrane bound mitochondrial protein.

Non-antibody ligands

Blotting techniques also have the potential of analyzing binding interactions other than those involving antibodies (examples are in Table V). Some of the surprising developments include the demonstration of specific binding of protein ligands to their receptors which had been separated on SDS gels, blotted, and shown to bind their respective ligands. For recovery of binding activity preservation of disulphide bridges and omission of the boiling step before electrophoresis seem to be important. Under these conditions proteins generally move faster; although molecular weight determinations are therefore less accurate, the presence or absence of different forms of receptor molecules can of course still be distinguished. Isoelectrofocussing or electrophoresis in none or mild detergents presumably would better preserve native structures, and therefore activities.

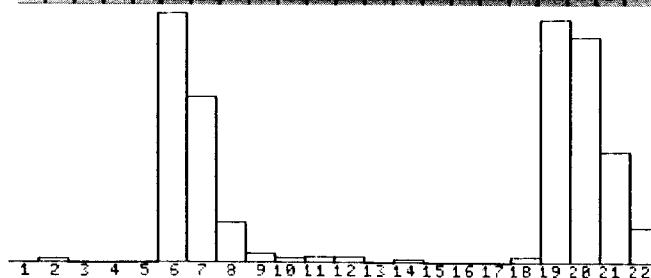
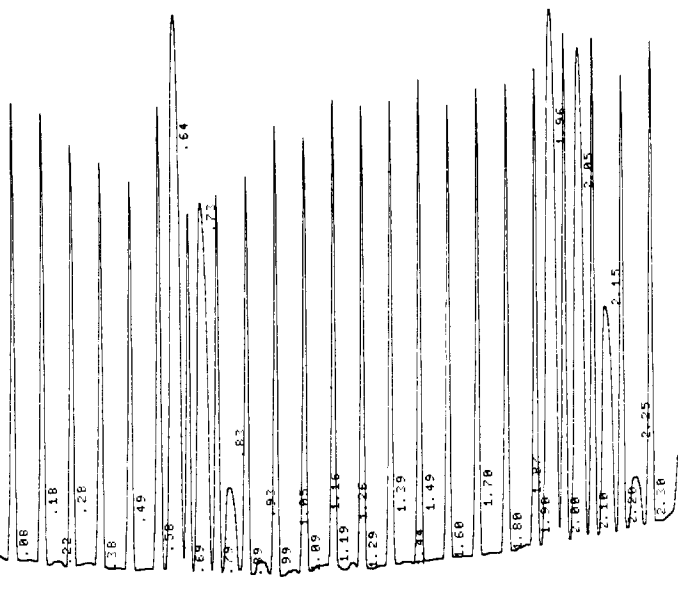
Comparison with alternative methodology

The obvious alternative to immunoblot analysis is the use of electrophoretic separation of immunoprecipitated, labelled antigens. In contrast to immunoblotting, the antigen has to be radioactively labelled, either biosynthetically or by post-labelling procedures. It must be soluble under conditions compatible with formation and precipitation of the specific immune complex. The proteins are likely to be in a conformation close to 'native'. Proteins naturally associated with the precipitated antigen may be co-precipitated but non-specific co-precipitation may also occur. The blotting techniques have the potential to analyze poorly soluble antigens and depending on the type of electrophoresis epitopes inaccessible on the native protein may be detected.

Swanson and Barrera (1983) studied interstrain cross-reactivity among gonococcal outer membrane protein II with rabbit sera against the isolated proteins. Extensive cross-reactions were observed by blot analysis. The same antiserum mainly recognized the homologous protein by immune precipitation. The findings suggested that these proteins share certain antigenic determinants accessible to antibodies only when denatured as on blots from SDS gels but inaccessible on the organisms surface. Similarly, Bolivar (1983) demonstrated the close relationship between 2 surface

Fig. 1. Computer program for organizing data from reflectance densitometer. The program is for the CAMAG TLC scanner interfaced to a Spectra Physics SP4100 computing integrator. A strip is illustrated between the corresponding analogue trace for the scan (top) and the histogram output option from the program. The program uses the original grid on the nitrocellulose as a reference grid, and prints out only peaks *not* corresponding to the grid lines. Data concerning the names of antigens in individual dots and the individual sera or antibody preparations are given in an input dialogue, and any combination of the output options selected. Information concerning the time between the preparation of the original dots and the assay itself is also automatically recorded. The position of the bars on the histogram can be related directly to the number of the antigen in the tabular format output. Details may be obtained from the authors.

INJECT TIME 00:34:03



PROJECT TITLE: DEMONSTRATION OF SCANNER PROGRAM
 DATE OF KIT: NOV. 17, 83
 DATE OF TEST: JAN. 06, 84
 STRIP NR. IS: 1.
 SERUM CODE IS: ASC. 7303
 *****DATA FOR NAMED PEAKS*****

NAME	PEAK HEIGHT	RT
1. BSA	0.	0.18
2. H2A	1.4	0.22
3. H2A/5	0.	0.38
4. H2A/25	0.	0.49
5. H2A/125	0.	0.58
6. H2B	130.7	0.64
7. H2B/5	87.2	0.73
8. H2B/25	20.4	0.83
9. H2B/125	3.7	0.93
10. H2B/625	1.5	1.05
11. H3	2.1	1.16
12. H3/5	2.2	1.26
13. H3/25	0.	1.39
14. H3/125	0.8	1.44
15. H4	0.	1.6
16. H4/5	0.	1.7
17. H4/25	0.	1.8
18. H4/125	2.1	1.87
19. TOTAL	127.5	1.96
20. TOTAL/5	118.4	2.05
21. TOTAL/25	58.1	2.15
22. TOTAL/125	17.8	2.25

TABLE V
EXAMPLES OF NON-ANTIBODY LIGANDS

Proteins blotted (property assayed)	Ligand	Reference
Nuclear matrix proteins from HeLa cells (DNA-binding proteins)	HeLa DNA	Bowen et al. (1980)
RSV-virion proteins (RNA-binding proteins)	RSV RNA	Bowen et al. (1980)
Histones H3 and H4 (histone-histone interaction)	H2a-H2b	Bowen et al. (1980)
Membrane proteins from A-431 cells (epidermal growth factor (EGF) receptor)	EGF	Fernandez-Pol (1982)
Crude membrane preparation from <i>Torpedo</i> electric organ (acetylcholine receptor)	Bungarotoxin	Gershoni et al. (1982b)
Cell lysate of chinese hamster ovary cells (attachment of gonococcal pili)	CNBr cleaved gonococcal pili	Gubish et al. (1982)
Brain membrane proteins (glycoproteins)	Concanavalin A	Hawkes (1982)
Frog virus 3 (FV) proteins and cell lysate from CHO cells (DNA-binding proteins)	FV 3 DNA	Aubertin et al. (1983)
Partially purified low density lipoprotein (LDL) receptor	LDL	Daniel et al. (1983)
Postmitochondrial supernate of rat pancreas (calmodulin-binding proteins)	Calmodulin	Gershoni and Palade (1983)
Liver plasma membrane (growth hormone receptor)	Human growth hormone	Haeuptle et al. (1983)
Thyroid plasma membrane (TSH receptor)	TSH	Islam et al. (1983)
Cell lysate of <i>E. coli</i> (λ repressor)	λ or total genomic DNA	Patel and Cook (1983)

proteins of *Paramecium* by cross-reaction of proteins blotted from SDS gels. These disulphide-rich proteins occur in conformationally different forms and do not cross-react in the native state.

Among 113 monoclonal antibodies binding to Herpes simplex virus 1-infected cells, Braun et al. (1983) found 7 antibodies reacting by immune precipitation only, 12 reacting with polypeptides on blots only, and 3 by both techniques. The number of different polypeptides in these 3 groups were 3, 6 and 1. In this system the diversity of monoclonal antibodies detected was larger using the immunoblot assay. The failure of the remaining majority of antibodies to react by either method was explained by poor solubility or denaturation by detergents.

Immunodetection after thin layer chromatography

Glycosphingolipids are antigenic molecules on the cell surface and may be used as markers for various cells and tissues. They can be analyzed by binding of antibodies after thin layer chromatography *in situ*. A very sensitive immunological procedure has recently been developed to detect glycosphingolipid antigens directly on the chromatograms (Brockhaus et al., 1981; Magnani et al., 1981). We recently reported

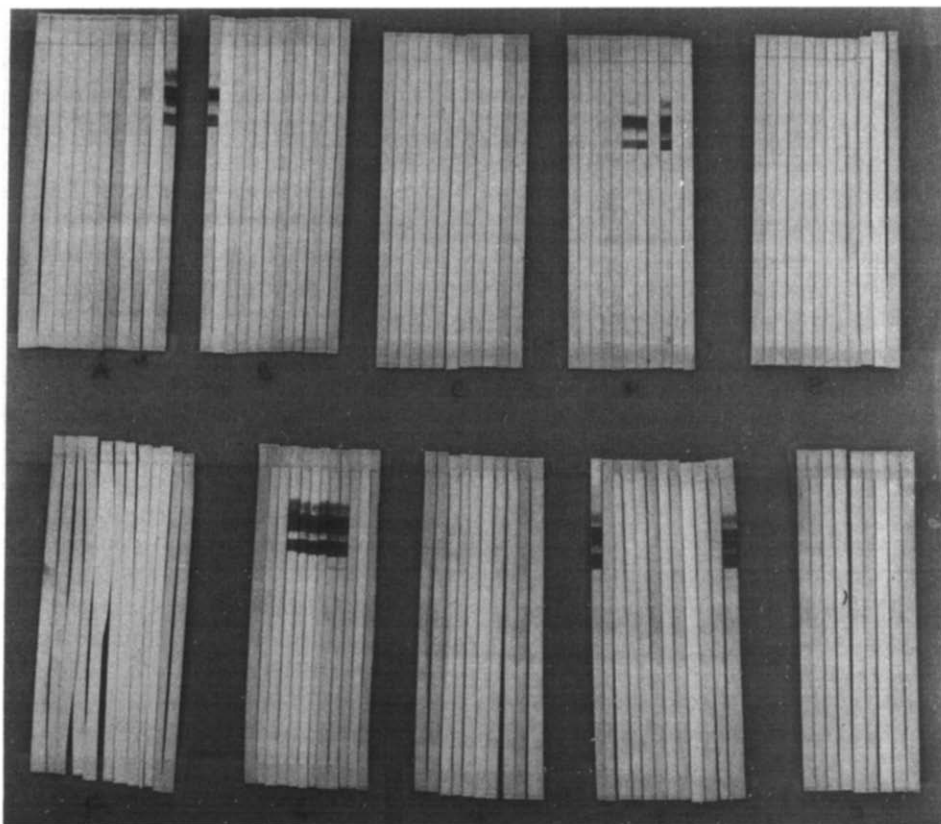


Fig. 2. Immunodetection of glycosphingolipids after blotting from thin layer chromatograms. Polar glycosphingolipids extracted from human granulocytes were applied as a line to high performance thin layer chromatography plates. After chromatographic development the TLC plate was dried and sprayed with isopropanol/water (2:1) until the plate was just visibly wet. A sheet of nitrocellulose covered with a sheet of polyethylene was immediately firmly pressed on the plate. After 1 min the sheet was removed and left to dry. The NC membrane was treated with diluted horse serum as for protein blots. The narrow strips were then individually incubated with moAbs raised against various lymphocytic cell lines and finally bound antibodies were visualized with a peroxidase conjugate. The few reacting moAbs stained each several bands. This staining was later shown to be reduced or abolished by lactofucopentaose III, an oligosaccharide present in human milk (Towbin, Uchanska-Ziegler, unpublished). The epitope, its characteristic sugar being fucose bound α 1-3 to *N*-acetylglucosamine seems to be present on several glycosphingolipids of different oligosaccharide chain length.

on a blotting assay for these molecules with enzyme-linked detection which is very suitable for screening purposes where many different antibody samples have to be tested against the same glycosphingolipid extract (Towbin et al., 1983; Towbin et al., 1984). The transfer to nitrocellulose of the developed glycosphingolipid pattern allows one to cut narrow strips which cannot be done with the brittle silica gel-coated plates. An example of a screen of monoclonal antibodies is shown in Fig. 2.

Applications of Immunoblotting

The number of publications using immunoblotting techniques is far too high to review comprehensively. In this section, we therefore selectively review only publications which appear to have some health of agronomical relevance.

Autoimmunity

In our original publication describing antibodies against ribosomal proteins we proposed the application of immunoblotting for characterization of antigens and screening sera in auto-immune (connective tissue) diseases (Towbin et al., 1979). Ribosomal antibodies were already known to be present in some auto-immune diseases (Schur et al., 1967). We demonstrated that immunoblots could be used for serological screening purposes, and for example demonstrated the presence of antibodies to ribosomal protein determinants (Gordon et al., 1982). In general, connective tissue diseases present a complex and perplexing serological picture. Several groups of investigators have begun to characterize relevant antigens. They have usually been defined by specific patient sera, and reactions of identity with reference sera (for a summary of the relevant antigens cf. Gordon and Rosenthal, 1984b). Some studies have now been carried out on some of these antigens by blotting. The molecular weights of the sero-positive peptides are summarized in Table VI. It can be seen that there is little concordance with respect to the molecular weights of these antigens. Clearly, more work is needed for the standardization of materials and procedures in order to obtain a more consistent picture. We anticipate that this work will offer powerful new tools for diagnosis, management and understanding the immuno-pathology of these diseases. To some of the antigens listed in Table VI (Sm, RNP and SSB) the corresponding antibodies can occur in systemic lupus erythematosus. In addition, antibodies against SNP are diagnostic of mixed connective tissue disease (Sharp et al., 1972), and antibodies against SSB of Sjögren's syndrome (Alspaugh and Tan, 1975). Anti-EBNA antibodies are characteristic for rheumatoid arthritis (Billings et al., 1983). Histones H1 and H2B have recently been shown to be major auto-antigens in systemic lupus erythematosus (Hardin and Thomas, 1984).

Auto-immune pathology may also be caused by cross-reactivity with bacterial antigens. Zabriskie and Friedman (1983) showed 2 protein bands (MW: 38,000 and 43,000) from heart muscle cross-reactive with antibodies present in streptococcal rheumatic fever sera. Cunningham and Russel (1983) observed a similar cross-reac-

TABLE VI
AUTO-IMMUNE ANTIGENS IDENTIFIED BY IMMUNOBLOTTING

References	M_r of immunoreactive peptides for			
	Sm	RNP	SSB	EBNA
White and Hoch, 1981	13 000	40 000 70 000		
Billings and Hoch, 1983	13 000	73 000		
	26 500			
	27 500			
Schrier et al., 1982	28 000			
Douvas, 1982	13 000	30 000		
Conner et al., 1982	16 000	19 000		
	25 000			
Wooly et al., 1983	17 000	68 000		
	26 000			
	27 000			
Bringmann et al., 1983	13 000	21 000		
	16 000	28 000		
	28 000	34 000		
	34 000	75 000		
	75 000			
Francoeur and Mathews, 1982			45 000	
Venables et al., 1983			29 000	
			40 000	
Billings et al., 1983				80 000

tion with rabbit sera raised against streptococcus A antigens.

There are 3 possible modes for clinical application of immunoblot methodology. (1) The patient's antibody is analyzed on blotted antigens thought to be related to the disease. (2) Specific antibodies (e.g. generated by immunization, monoclonals etc.) may be evaluated on blotted pathological antigens. (3) The patient's immunoglobulins themselves can be analyzed; for example, for polyclonality by isoelectric focussing and blotting, followed by probing with specific antigens or immunoglobulins. Examples of all 3 modes have been published and are indicated in the literature summary in Table VII by (1), (2) or (3).

Since the nitrocellulose with bound antigens forms a stable, dry reagent, in principle, the blot itself could become part of a routine immunodiagnostic test. With this in mind, we have also screened the literature for such possible direct applications. When such an application seems conceivable, we have indicated this in Table VII by a (4).

Infections

The immune response to worm infestation was previously difficult to study because of its complexity and the difficulty of obtaining solubilized antigen preparations. These problems are being solved by immunoblotting (Tsang et al., 1982; Weiss

TABLE VII
APPLICATION OF IMMUNOBLOTTING TO ANALYSIS OF PATHOLOGICAL CONDITIONS

	References	Comments ^a
<i>Worms</i>		
<i>Dipetalonema</i>	Lucius et al., 1983	1,2
<i>Schistosoma</i>	Tsang et al., 1982	2
<i>Onchocera</i>	Weiss et al., 1982	1
<i>Echinococcus</i>	Gottstein, 1983	1
<i>Protozoa</i>		
<i>Amoeba</i>	Kettis et al., 1983	1
<i>Plasmodium</i>	Anders et al., 1983	1
	Coppel et al., 1983	2
	Freeman and Holder, 1983	2
	Holder et al., 1983	2
<i>Toxoplasma</i>	Erlich et al., 1983	1,4
	Sharma et al., 1983	1,4
	Partanen et al., 1983	1,4
<i>Mycoplasma</i>	Wise and Watson, 1983	2
<i>Spiroplasma</i>	Townsend and Archer, 1983	2
<i>Chlamydia</i>	Stephens et al., 1982	2
	Newhall et al., 1982	1,4
	Saikkku et al., 1983	1,4
	Caldwell and Schachter, 1983	2
	Matikainen and Terho, 1983	2
<i>Bacteria</i>		
<i>Neisseria</i>	Swanson and Barrera, 1983	2
	Gubish et al., 1982	
	Swanson et al., 1982	2
<i>Treponema</i>	Lukehardt et al., 1982	2
	Walfield et al., 1982	1
	Hanff et al., 1983a	2
	Hanff et al., 1983b	1,4
	Baughn et al., 1983	2
<i>Spirochetes</i>	Burgdorfer et al., 1983	1
	Barbour et al., 1983a	1
	Barbour et al., 1983b	2
<i>Mycobacterium leprae</i>	Chakrabarty et al., 1982	1
	Gillis and Buchanan, 1982	2
	Miller et al., 1983	1
	Ivanyi et al., 1983	2
<i>Bacteroides</i>	O'Donnel et al., 1983	2
	Cousland and Poxton, 1983	2
<i>Viruses</i>		
<i>Measles</i>	Hall and Choppin, 1981	1
	Karcher et al., 1981	2
<i>Herpes</i>	Norrild et al., 1981	2
	Braun et al., 1983	2
	Eberle and Mou, 1983	1
	Palfreyman et al., 1983	2

TABLE VII (continued)

	References	Comments ^a
Herpes encephalitis	Vaheri et al., 1982	3
Cocksackie	Dörries and TerMeulen, 1983	1
Hepatitis B	McMichael et al., 1981	2
	Gupta, 1982	2
Epstein-Barr virus	Strnad et al., 1981	2
Poliovirus	Blondel and Crainic, 1981	2
	Thorpe et al., 1982	2
	Brioen et al., 1982	2
Cytomegalovirus	Weiner and Gibson, 1981	2
Baculovirus	Smith and Summers, 1981	2
	Naser and Miltenburger, 1982	2
	Knell et al., 1983	2
	Naser and Miltenburger, 1983	2
	Hohmann and Faulkner, 1983	2
Bovine	Roseto et al., 1982	2
Plant virus	O'Donnel et al., 1982	2
	Rybicki and Von Wechmar, 1982	2
<i>Transformed state</i>	Suni et al., 1981	2
	Nigg et al., 1982	2
	Du Bois and Rossen, 1983	1
	Rordorf et al., 1983	1
	Rosen et al., 1983	2
	Takahashi et al., 1983	2
	Rittenhouse et al., 1984	2
<i>Myeloma proteins</i>	Melcion et al., 1983	3
<i>Genetic variants</i>		
Complement C1q	Chapuis et al., 1982	2
Hypoxanthine-guanine phosphoribosyl transferase	Wilson et al., 1982a	2
Adenine phosphoribosyl transferase	Wilson et al., 1982b	2
Low-density lipoprotein receptor	Beisiegel et al., 1982	2
<i>Phenotypic variants</i>		
β -Hexosaminidase	Zühlsdorf et al., 1983	2
Prolactin, growth hormone	Meuris et al., 1983	2
Insulin	Kakita et al., 1982	2
<i>Neuropathy</i>		
Multiple sclerosis	Newcombe et al., 1982	2
	Walker et al., 1983	4
	Page et al., 1983	1
Peripheral neuropathy	Latov et al., 1981	2

TABLE VII (continued)

	References	Comments ^a
<i>Allergy</i>	Weiss et al., 1982	1
	Sutton et al., 1982	1
	Peltre et al., 1982	1
<i>Ageing</i>	Coppard et al., 1982	1
<i>Fertility</i>	Edwards et al., 1981	1
	Lee et al., 1982	1

^a Comments:

- (1) use of blotted antigens to determine patient antibody profile;
- (2) use of blot in characterization of specificity of antibodies, including monoclonals, to be used as immunological probes for pathological antigens;
- (3) blotting of immunoglobulins to determine class specificity and/or oligo-clonality;
- (4) possible use of blot itself as part of diagnostic test.

et al., 1982; Lucius et al., 1983). This work may lead to better methods for preparing antigens or specific antibodies for early diagnosis. Gottstein (1983) has shown improved serological distinction between different *Echinococcus* infections.

Protozoan infestation is responsible for diverse diseases such as amoebic dysentery, malaria, toxoplasmosis. Kettis et al. (1983) have demonstrated the serological response to *Entamoeba histolytica* with immunoblotting, and this may lead to better serodiagnosis of extra-intestinal infections. Anders et al. (1983) have serologically investigated a peptide which is secreted in large amounts by the malarial *Plasmodium*. This might be the basis by which the host immune system is overwhelmed. The *Plasmodium* of malaria also evades the host immune system by its high genetic variability. Immunoblotting has proved to be an important technique for the analysis of this disease. Coppel et al. (1983) found that antibodies raised against a peptide containing a characteristic repeating sequence of amino acids react with a 220,000 molecular weight polypeptide of the *Plasmodium*, which is, however, isolate specific. Further, Holder et al. (1983) have shown that, while monoclonal antibodies against a peptide of this molecular weight are species specific, a polyclonal antibody is species cross-reactive. They also demonstrated the processing pathway of this peptide and its secretion (Freeman and Holder, 1983). Erlich et al. (1983), Sharma et al. (1983) and Partanen et al. (1983) have all investigated the serological profile in the immune response to toxoplasmosis. The blot reveals immune response to peptides which are diagnostic of toxoplasmosis.

Monoclonal antibody probes are being developed for *Mycoplasma* typing and immunodiagnosis. Wise and Watson (1983) have developed an antibody which is specific for a *Mycoplasma* antigen expressed on the surface of the infected cell.

Immune response against specific polypeptides has been analyzed in chlamydial infections (Newhall et al., 1982; Saikku et al., 1983). This response may provide a more precise diagnosis than the total chlamydial antibody determination. Further, Stephens et al. (1982), Caldwell and Schachter (1983) and Matikainen and Terho

(1983) have developed specific antibody probes which might be of diagnostic potential. They have used immunoblotting to characterize their antibodies.

Neisseria gonorrhoeae outer membrane structure is being studied with polyclonal (Swansson et al., 1982) or monoclonal (Swansson and Barrera, 1983) antibodies. Gubish et al. (1982) used immunoblots of host proteins for identifying receptors for gonococcal pili. The immune response to *Treponema* infection is complex, and characteristic for the stage of the disease. Diagnostic assays have been based on preparations of the non-pathogenic Reiter strain. Hanff et al. (1983a) and Lukehardt et al. (1982) have determined the immunogenic peptides common to this and the pathogenic Nicols strain. In a study by Hanff et al. (1983b) immunoblots served to determine the profile of the immune reaction during the course of infection. In relation to the question as to whether the circulating immune complexes in syphilitic infection contain auto-antigens or *Treponema* antigens, immunoblotting has been used to identify the treponemal antigens present in the complexes (Baughn et al., 1983). Walfield et al. (1982) have used immunoblots to verify the product made in recombinant *E. coli* under the direction of *Treponoma* genes.

Immunoblotting has been used extensively to elucidate the etiology of tick borne spirochete infections. Antigenic identity was shown between the antigen present in the patient and the spirochete isolated from the tick, using the patient's serum (Barbour et al., 1983a) and a monoclonal antibody (Barbour et al., 1983b). Immunoblotting was also used to present evidence for the identity between the Lyme disease described in the U.S. and erythema chronica migrans from Switzerland (Burgdorfer et al., 1983).

Patient's immune response in leprosy has been characterized by immunoblotting using the *Mycobacterium leprae* antigens (Chakrabarty et al., 1982) or a heterologous polysaccharide antigen (Miller et al., 1983). The specificity of monoclonal antibodies has also been determined by immunoblot technique (Gillis and Buchanan, 1982; Ivanyi et al., 1983). These may provide useful diagnostic reagents for early detection and epidemiological survey.

Immunoblot analysis finds its most obvious application in serological analysis of viral infections, since the organisms are difficult to cultivate and are antigenically complex. They are also often poorly soluble and therefore difficult to apply in classical methods. Karcher et al. (1981) gave the example of measles, and Norrild et al. (1981), Palfreyman et al. (1983) and Braun et al. (1983) of herpes simplex. Immunoblotting has been used to distinguish the profiles of antibodies against individual herpes simplex polypeptides in normal individuals and individuals with persistent lesions (Eberle and Mou, 1983).

With immunoblot detection, Hall and Choppin (1981) showed the selective absence of a single measles virus peptide in the brain of individuals with subacute sclerosing panencephalitis, supporting the idea of defective virus production. Dörries and TerMeulen (1983) have identified an IgM antibody directed against a specific peptide which occurs in Coxsackie infection. Blotting has also been used to investigate the nature of the antigen in hepatitis B. McMichael et al. (1981) correlated changes in serum HBs antigen electrophoretic mobility with patient status, and Gupta (1982) investigated the HBs in circulating immune complexes. Further

examples are poliovirus (Blondel and Crainic, 1981; Brioen et al., 1982; Thorpe et al., 1982) and cytomegalovirus (Weiner and Gibson, 1981). Immunoblot analysis also provides a realistic method for identifying plant viral antigens in field isolates, and numerous examples have been described (O'Donnel et al., 1982; Rybicki and Von Wechmar, 1982). Possible veterinary applications originate from studies on bovine virus studies (Roseto et al., 1982).

Extensive studies of insect virus antigens, of possible use in pest control, have been performed (Smith and Summers, 1981; Naser and Miltenburger, 1982, 1983; Hohmann and Faulkner, 1983; Knell et al., 1983).

Transformed state

Work has also been carried out on antigens related to the transformed state. Strnad et al. (1981) characterized the Epstein-Barr virus nuclear antigen, which is present in Epstein-Barr virus transformed cells and showed association with transformed cell lines. Billings and Hoch (1983) showed identity between an 80,000 M_r antigenic peptide in both the rheumatoid arthritis nuclear antigen and the Epstein-Barr virus nuclear antigen (see also Table VI). Antibodies raised against protein from expression of fused genes of *E. coli* β -galactosidase and the Epstein-Barr genome were found to recognize 2 sets of nuclear antigens in Epstein-Barr transformed cell lines (Hennessy and Kieff, 1983). Further, immunoblot analysis has been valuable to characterize tumour-associated antigens (Rosen et al., 1983; Takahashi et al., 1983). Suni et al. (1981) showed the presence of antibodies to retroviral protein p30 in cord blood sera, which is correlated with problems in pregnancy, and we identified the same antibody in auto-immune mice (Rordorf et al., 1983). The carcinoembryonic antigen, which is widely used in cancer diagnosis and management, can only be visualized after electrophoresis by immunoblotting (Rittenhouse et al., 1984). Immunoblotting has also been used to characterize myeloma proteins from unpurified urine in monoclonal gammopathy (Melcion et al., 1983). Nigg et al. (1982) investigated the nature of cross-reactions observed with monospecific rabbit antibodies directed to a synthetic heptapeptide corresponding to the carboxyterminal sequence of p60^{src}. By indirect immunofluorescence microscopy and blotting techniques cytoskeletal proteins were identified as major cross-reactive targets in untransformed cells. These cross-reactions could be inhibited with soluble peptide in both assays and most likely were due to shared amino acid sequences.

Electrophoretic variants

Immunoblot analysis has also been used for detecting electrophoretic variants of immunologically cross-reacting proteins. Thus, genetic variants with modified activities have been detected for complement C1q (Chapuis et al., 1982), hypoxanthine ribosyl transferase (Wilson et al., 1982a), adenine phosphoribosyl transferase (Wilson et al., 1982b) and low density lipoprotein receptor (Beisiegel et al., 1982). Further, phenotypic variations of precursor and mature form of β -hexosaminidase and cathepsin D in serum or urine were investigated in hepatitis and nephritis (Zühlendorf et al., 1983). Previously unknown higher molecular weight cross-reacting species of growth hormone and prolactin were detected in pituitary extracts (Meuris

et al., 1983). No insulin precursors were detectable in pancreas extracts (Kakita et al., 1982).

Neuropathy

Determination of immune parameters in cerebrospinal fluid is of interest in neuropathological disorders. Polyclonality of immunoglobulins in cerebrospinal fluid can be determined from unpurified cerebrospinal fluid, and is more rapid than preceding methodology (Walker et al., 1983). This methodology has been applied to multiple sclerosis and related disorders (Glynn et al., 1982; Keir et al., 1982; Walker et al., 1983), and in herpes simplex viral encephalitis (Vaheri et al., 1982). Page et al. (1983) showed the absence of antibodies to myelin basic protein in cerebrospinal fluid of multiple sclerosis. Newcombe et al. (1982) surveyed possible protein changes in multiple sclerosis and subacute sclerosing panencephalitis. Latov et al. (1981) identified myelin antigens which react with circulating antibodies in plasma cell dyscrasia and peripheral neuropathy and Braun et al. (1982) for polyneuropathy.

Allergy

Allergy is an immunological response to complex environmental agents. Diagnosis puts demands on immunoassay technique because of the requirement to determine IgE antibodies which might be present in serum at far lower concentrations than other immunoglobulins. Sutton et al. (1982) have surveyed cereal grain allergens in connection with baker's asthma, and Peltre (1982) has investigated pollen allergens. Furthermore, Weiss et al. (1982) have determined profiles of IgE reactivity in helminth infestation.

Applications of Dot Immunobinding

Dot immunobinding is attractive for routine diagnostic application because the procedure permits the performance of dozens of assays on 1 assay strip and detects antibodies with high sensitivity. With suitable developing antibodies the assay can easily be made immunoglobulin-class specific, as is often required for diagnostic purposes. In our original publication (Hawkes et al., 1982) we demonstrated the feasibility of such serological screening with a variety of antigens from commercially available kits.

Further experience of dot immunobinding has now been obtained with a set of antigens derived from a standard block of complement fixation assays provided by a commercial clinical laboratory for respiratory infection diagnosis: adenovirus, *Chlamydia*, influenza A, influenza B, parainfluenza 1, 2 and 3, mumps, *Mycoplasma*, a mixture of picorna viruses, Q fever and respiratory syncytial virus (Gordon, Holliger and Viollier, unpublished).

We have further developed assays for total IgE and 2 allergens (dust mite and bee venom) (Derrer et al., 1984), and shown that the system is specific for IgE and sufficiently sensitive for determinations in serum. The assay is reproducible with coefficients of variation between 3 and 6%, and correlates with commercial assays

for total IgE and the allergen-specific IgE. Walsh et al. (1984) have developed a similar system for qualitative use with several allergens deposited on nitrocellulose discs.

We have established an auto-immunity profile for connective tissue diseases which measures anti-DNA antibodies, rheumatoid factor and antibodies against subcellular fractions from HeLa cells (Gordon and Rosenthal, 1984b). This assay correlated well with commercial tests for rheumatoid and anti-nuclear factor and with clinical diagnoses. The assays were standardized with international reference sera, and showed self-consistent performance in successive samples from the same patient.

Pappas et al. (1983) applied dot immunobinding to visceral leishmaniasis. They performed assays with serial dilutions of sera with single dots in microtitre plate wells, and showed that, in spite of cross-reactivity with African trypanosomiasis, Chagas' disease and lupus erythematosus, the tests were diagnostically useful and lend themselves especially well to field screening. They also found far less antigen was required per test than in a comparable enzyme immunoassay. In the same publication, they cite their own unpublished work with similar assays for hydatid disease and coccidioidomycosis.

Concluding Remarks

Porous membranes have proven to be convenient supports in solid-phase immunoassays for many particulate or soluble antigens. Non-isotopic dot immunobinding assays show great promise for application as diagnostic tests in a variety of areas and may replace many existing standard tests. The possibility of testing a serum against hundreds of antigens in 1 operation provides new aspects in immune diagnosis. Immunoblotting is by now well established as a tool in immunobiochemical analysis. New findings with immunoblot methodology are likely to be made in the future. These may be the detection of pathological electrophoretic variants; the detection of presence or absence of pathological immunoreactivity amongst electrophoretically separated antigens; or changes in patient antibody profile to electrophoretically separated antigens. These kinds of analyses may well find applications in routine diagnostics.

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