Immunoblotting and the immune response to leprosy

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Pathogenic mycobacteria are an important cause of human disease (leprosy and tuberculosis) affecting over 30 million individuals worldwide. During infection, mycobacteria live within phagocytic cells of the immune system and protective immunity involves recognition of mycobacterial antigens by T-lymphocytes. Infection also results in generation of an antibody response to the pathogen and monitoring of antibody levels is one of the most useful techniques in the diagnosis and management of leprosy patients (Anon, 1985). The combination of specific antibody and antigen detection provides a technically convenient procedure which allows diagnosis, by antibody and antigen detection, and also to analysis of the T-cell response to mycobacterial infection.

Glycolipid blotting: the phenolic glycolipid of M. leprae

Most individuals develop antibodies to common non-pathogenic environmental mycobacteria. In order to develop specific serological tests for mycobacterial diseases, it is therefore necessary to identify antigens which are unique to the pathogenic strain. Mycobacteria produce a surface capsule consisting mainly of complex lipid components, some of which are species-specific antigens (Brennan, 1984).

In the case of M. leprae, a phenolic glycolipid possessing a specific trisaccharide moiety has been identified in infected tissues from patients (Young, 1981) and experimental animals (Hunter & Brennan, 1981). Antigenicity of the phenolic glycolipid has been demonstrated using human sera and mouse monoclonal antibodies (Cho et al., 1983; Young & Buchanan, 1983; Young et al., 1984). Immunoblotting represents a useful approach to determine the specificity of antibody responses to the M. leprae phenolic glycolipid.

Immunoblotting on t.l.c. plates. Lipids are conveniently fractionated by t.l.c. on silica gel plates and several authors have described procedures for reacting antibodies with glycolipids separated in this way. Such an approach was pioneered by Magnani et al. (1981, 1982) using iodinated antibody reagents and the same technique has been applied for enzyme-linked detection (Harpin et al., 1985). Silica plates (on glass or foil supports) can be directly incubated with antibody reagents in aqueous solutions without the necessity of any transfer or fixation procedure for the insoluble glycolipid antigens. Commercially produced t.l.c. plates with strong organic binders and firmly fixed silica coating have appropriate physical properties for such assays. Lower antigen concentrations are required when 'high-performance' t.l.c. plates (coated with smaller particle size silica) are used, possibly because a relatively higher proportion of glycolipid is exposed on the surface of the silica particles rather than 'hidden' inside pores. Blocking of non-specific binding of antibodies to the t.l.c. plate can present problems in that, for some glycolipids, use of detergents can cause antigen solubilization, and a procedure of coating plates with polysobutylmethacrylate has been found generally useful (Magnani et al., 1982). The efficiency of this type of direct immunoblotting, in terms of sensitivity and 'signal-to-noise' ratio, is a function of the physical properties of the individual glycolipid and also of the affinity of the antibody used.

Partial hydrolysis of the M. leprae phenolic glycolipid under acid or alkaline conditions results in generation of several degraded forms of the molecule lacking particular fatty acid or sugar components. These can readily be separated by t.l.c. Subsequent immunoblotting with monoclonal antibodies shows that the deacylated molecule retains antigenicity, but that disruption of the carbohydrate moiety destroys antibody binding. By this approach the terminal sugar residue (3,6-dioxymethylglucose) was identified as the key factor in antibody recognition of the M. leprae glycolipid (Young et al., 1984).

Immunoblotting on membranes. The phenolic glycolipid is a relatively non-polar glycolipid and does not perform well during direct t.l.c. plate immunoblotting. Other methods for blotting glycolipids, such as transfer to a nitrocellulose membrane, have been developed and may be more suitable for particular antigens (Towbin et al., 1984). The property of nitrocellulose which has led to its success in immunoblotting is its tight binding of protein antigens. This same property can, however, lead to problems with non-specific binding of antibody reagents. Since the ability to bind proteins is not required for a support material to be used with glycolipids, it is possible to substitute a membrane specifically designed to minimize protein binding in order to decrease background reactions with antibodies. Polysulphone membranes have the appropriate ability to bind glycolipids and to resist non-specific protein binding (Young et al., 1985a,b).

The M. leprae phenolic glycolipid can be readily detected in dot-blot assays after spotting on to polysulphone from a solution in hexane. This type of assay has been adapted as a simple spot test for detection of specific immunoglobulin M antibodies in sera from leprosy patients and their household contacts (Young et al., 1985b).

It is also possible to carry out lipid fractionation by liquid chromatography directly on the polysulphone membrane (Young et al., 1984). As in the case of direct t.l.c. blotting, therefore, this technique shares the key feature with Western blotting of proteins in combining a biochemical fractionation step with a test for immunological specificity. Lipid chromatography on a polysulphone support has been used in development of an assay for detection of phenolic glycolipid in serum or urine samples from leprosy patients (Young et al., 1985c). The combination of specific antibody and antigen detection techniques provides an important new tool in the clinical management of leprosy patients (Anon, 1986).

Immunoblotting of antigens recognized by T-cells

While antibodies have the ability to bind directly to antigen molecules, the specific antigen receptor on T-lymphocytes recognizes antigens only after they have been 'processed' and are exposed on the surface of an antigen-presenting cell in combination with a component of the major histocompatibility complex (an MHC or HLA molecule). Since protective immunity to mycobacterial disease involves antigen recognition by T-cells, it is of interest to apply immunoblotting in this context.

Conventional nitrocellulose blots prepared from SDS/polyacrylamide gels (Towbin et al., 1979) can be used in T-cell proliferation assays after cutting into sections corresponding to different molecular mass proteins (Young & Lamb, 1986). Prior conversion of nitrocellulose strips to a microparticulate form by treatment with dimethylsulphoxide provides a technically convenient procedure which allows a

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blot from a single gel to be used in multiple proliferation assays (Abou-Zeid et al., 1987). Presumably, degradative enzymes within the antigen-presenting cells are capable of digesting and processing a portion of the antigens from the nitrocellulose membrane. Using this approach it has been possible to identify polypeptides involved in T-cell recognition of complex antigen mixtures, such as a mycobacterial extract, by monitoring the proliferative response of clonal or polyclonal T-cell populations (Lamb & Young, 1987).

An analogous approach can be used to demonstrate recognition of recombinant antigens by T-cell clones after addition of plaque immunoblots to T-cell proliferation assays (D. B. Young & J. R. Lamb, unpublished work).

Summary

Immunoblotting has provided a powerful and effective approach to dissection of the immune response to mycobacterial antigens in a situation in which the availability of isolated antigenic components is severely limited. The basic approach of blotting onto to a solid-phase support has been used in combination with SDS/polyacrylamide-gel electrophoresis, t.l.c., recombinant DNA technology and T-cell cloning in order to carry out a comprehensive analysis of glycolipid and protein antigens involved in the immune response to mycobacterial infection.


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Blotting analysis of adhesive proteins: an evaluation of the technique using B16F10 malignant melanoma cells

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The identification of adhesive molecules can proceed in several different ways, but one of the most convenient involves the adsorption of a putative adhesive molecule on to an artificial substrate (such as tissue culture plastic) followed by probing the adsorbed material with a suspension of cells. In the absence of protein in the suspending medium, most cells adsorb non-specifically to tissue culture plastic and thus any sites not occupied by the adsorbed adhesive molecules under test must be blocked with a non-adhesive agent such as albumin. The major problem of identifying an adhesive protein, however, is not so much in showing that cells adhere to it, but more in how to identify it from a complex mixture of molecules which may have been extracted from whole cells or multicomponent substrates. Within a mixture of molecules, some may block adhesion by competing for substrate adsorption with the adhesive molecule, while others may interact with the molecule in question to effectively neutralize its adhesive properties. Some of these problems may be overcome if the mixture can be fractionated and the fractions tested in an adhesion assay. In the original report on cell blotting (‘bioautography’) by Klebe et al. (1978), 50–100% (w/v) serum was electrophoresed on cellular acetate and a diffusion replica was made by overlaying on to a gel of 0.25% (w/v) type I collagen. Material which did not bind to the collagen was eluted by washing, and any adhesive molecules were then identified by probing with 4 × 10⁷ CHO cells followed by fixing, and then staining with 0.1% (w/v) Toluidine Blue. Using this technique, two adhesive bands were identified in serum: one by its isoelectric point (approx. pl 4.8) and the other by its extremely large size (failing to run into the gel). The main limitation of this study was associated with restriction of the analysis to those molecules which bound to collagen under the conditions of the experiment. Hayman et al. (1982) improved upon this technique by first depleting plasma of albumin and immunoglobulin G, and then by separating the remaining proteins using SDS/polyacrylamide-gel electrophoresis (PAGE) under reducing conditions. A replica of the electrophoretic pattern was then made by diffusion blotting of the separated molecules on to a nitrocellulose filter according to the method of Bowen et al. (1980). After blotting, the filter was blocked with 5 mg of bovine serum albumin/ml, probed with NRK cells (10⁷ cells


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