REVIEW

IMMUNOFLUORESCENCE OF THE IMMUNOBULLOUS DISORDERS. PART ONE : METHODOLOGY

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In the past two decades the immunofluorescence techniques have greatly contributed to the diagnosis, treatment and understanding of the immunobullous disorders. The methodology of direct and indirect immunofluorescence as applied to the immunobullous disorders is described in detail.

Key Words : Immunofluorescence, Split skin technique, Immunobullous disorders

Introduction

Immunofluorescence techniques are essential to supplement clinical findings and histopathology in the diagnosis of the immunobullous disorders. These rapid and reliable techniques permit early diagnosis and treatment of these potentially life-threatening disorders. They are also useful in the subsequent monitoring of disease activity in some disorders. Occasionally, immunofluorescence techniques will need to be supplemented by immunoelectron microscopy or immunoblotting for definitive diagnosis. In addition to being extremely useful clinical investigations, the immunofluorescence methods are used in research to advance the understanding and classification of the immunobullous disorders.

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Skin Biopsy

Direct immunofluorescence studies require a biopsy of the patient's skin or mucosa. The choice of biopsy site is very important. Biopsies of the lesions themselves are not satisfactory as immunoreactants and tissue structures may be altered, making interpretation difficult. Ideally, both clinically unaffected and immediately perilesional skin should be biopsied. Perilesional skin from the end of an elliptical biopsy taken for histopathology (Fig. 1) and a 3 mm punch biopsy of uninvolved skin are adequate specimens. In some of the blistering disorders one of these alone will be sufficient. Table I shows the preferred biopsy site for each of the disorders.

Ideally, tissue specimens are collected and snap-frozen immediately. In most cases, facilities for direct immunofluorescence are not available and tissue specimens will need to be transported to the laboratory. In this situation, biopsies should be washed in distilled water or normal saline and placed in Michel's liquid fixative.[1] This fixative contains proteolytic enzyme inhibitors, preventing autolysis of tissue structures and immunoreactants. Washing of the tissue removes blood proteins

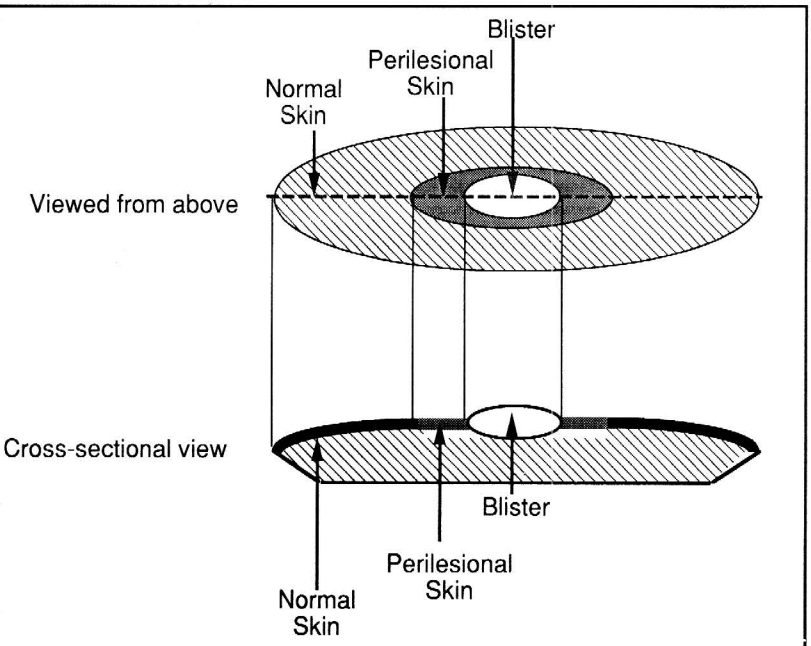

Fig 1 Skin biopsy for IF studies

Tabel I: Biopsy sites for direct immunofluorescence.

Condition	Biopsy site(s)
Pemphigus (all types)	Perilesional and uninvolved (buttock)
Pemphigoid (all types)	Perilesional and uninvolved (thigh or arm)
Pemphigoid gestationis	Perilesional
Linear IgA disease	Perilesional and univolved (buttock)
Epidermolysis bullosa acquisita	Perilesional
Bullous systemic lupus erythematosus	perilesional
Dermatitis herpetiformis	Uninvolved

and ensures maintenance of a neutral pH in the medium. This will ensure immunoreactant preservation at ambient temperatures for up to 6 months for standard immunofluorescence[2] and up to 4 weeks for immunoelectron studies (unpublished data). Thus, specimens may be mailed to the laboratory from a distance. Michel's medium itself may be stored at room temperature for one year without any deterioration.

In the laboratory, tissue specimens received in Michels' medium are washed in phosphate buffered saline (PBS) to remove ammonium salts and any residual blood proteins. Washing is carried out by immersion in a bath of the buffer which is continuously stirred with a magnetic stirrer. The tissue is then snap-frozen. A drop of OCT embedding compound on a piece of cork is briefly immersed in a cold hexane bath until the peripheral OCT is frozen. The skin biopsy is then correctly orientated in the central fluid portion, covered with further OCT and briefly returned to the bath. The frozen skin biopsy in a shell of OCT is then stored in liquid nitrogen. This method reduces freezing artefacts and

properly orientates the biopsy for subsequent sectioning.

Blood Specimens

Samples of serum are required for indirect immunofluorescence techniques to detect circulating autoantibodies. Approximately 10 ml of blood should be collected in a tube without anticoagulant and transported to the laboratory within 48 hours. Serum is then separated from the clot by centrifuging and stored at -25^0 C until analysis is performed. Alternatively, separated serum may be sent to the laboratory within one month of collection.

Immunofluorescence Techniques

I Direct Immunofluorescence

Direct immunofluorescence (DIF) is a one-step procedure used to detect and localize immunoreactants deposited in vivo in the patient's skin or mucosa. The immunoreactants include antibodies, complement components and fibrinogen.

Tabel II: Patterns of immunoreactant deposition

A Intercellular Substance (ICS)	
IgG	Pemphigus vulgaris (PV) Pemphigus foliaceus (PF) Paraneoplastic pemphigus (PNP)
IgG-ICS+BMZ	Pemphigus erythematosus (PE) Paraneoplastic pemphigus (PNP)
IgA	IgA pemphigus (IAP)
B Basement Membrane Zone (BMZ)	
IgG+/or C3	Bullous pemphigoid (BP) Cicatricial pemphigoid (CP) Pemphigoid gestationis (PG) (Epidermolysis bullosa acquisita (EBA)) (Bullous systemic lupus erythematousus (BSLE))
IgG, IgA, IgM, C3	Epidermolysis bullosa acquisita (EBA) Bullous systemic lupus erythematosus (BSLE) Cicatricial pemphigoid (CP) (Bullous pemphigoid (BP)) (Pemphigoid gestationis (PG))
IgA +/- C3	Dermatitis herpetiformis (DH) Liner IgA disease (LAD)

Frozen sections 5μm in thickness are cut with the cryotome and placed on slides. These are dried for ten minutes with an electric fan. Inadequate drying of sections between processing steps may lead to their detachment during washing. The sections are then washed in PBS at a pH of 7.4 for ten minutes to remove surrounding OCT compound. The sections are fan-dried once more and incubated with monospecific fluorescein isothiocyanate (FITC)-labelled antisera for thirty minutes at 37^0 C. Antisera to IgG, IgA, IgM, fibrinogen and the C3 component of complement should be routinely employed. Antisera to particular subclasses of immunoglobulins and other components of complement are also available but are less commonly used. Sensitivity and specificity of staining may be maximized by the use of the optimal dilution of the labelled antisera. This is determined by a chess-board titration procedure utilizing a known positive tissue specimen.[3] The sections are washed in PBS to remove unbound antisera, fan-dried and mounted in a drop of buffered glycerol. They are then viewed with the fluorescence microscope.

The distribution and type of immunoreactant deposition is recorded. The class and subclass of immunoglobulins and the presence or absence of complement is noted. Excessive fibrin depostion indicates that immunoreactants have been present more than 24-48 hours. Immunoreactants are deposited in two main patterns: in the epidermal intercellular space (ICS) and along the basement membrane zone (BMZ).

Intercellular space immunoreactants may be found throughout the epidermis or restricted to certain layers. Basement membrane zone deposits may be smooth and linear, granular and discontinuous or a combination of the two. The patterns of immunoreactant deposition in the various bullous disorders are shown in Table II.

A number of artefacts must be differentiated from significant findings.[3,4] Autofluorescence by proteins such as keratin and elastic fibres may be a significant problem. Non-specific staining results from binding of eosinophils. Undesired specific staining is produced by antibodies with unwanted specificities, leading to binding of the fluorescein conjugate to irrelevant structures.

A trained observer with knowledge of dermatopathology and immunofluorescence will gain considerable assistance from simultaneous examination of a haematoxylin and eosin stained frozen section to aid in identification of fluorescing structures.

II Indirect Immunofluorescence

Indirect immunofluorescence (IIF) is a two-step procedure used to identify circulating autoantibodies to cutaneous or mucosal stuctures in a patient's serum. These antibodies are most commonly of IgG or IgA classes.

In the first step, serial dilutions of the patient's serum in PBS are incubated with frozen sections of the substrate. At least two 5 μm thick sections are prepared by being alternately fan-dried, washed in PBS and fan-dried for ten minutes each. The initial serum dilution to 1:10 or 1:80 is incubated with the sections for thirty minutes at 37° C. If positive, subsequent incubations are used with increasingly higher dilutions of sera. Autoantibodies in the serum bind to components of the epidermis and basement membrane zone. Three washings of ten minutes each in PBS are carried out to remove unbound serum.

In the second step, the bound autoantibodies are labelled with fluorescein isothiocyanate (FITC)-conjugated anti-human immunoglobulins. Class-specific antibodies are routinely used but subclass-specific antibodies are also avaible. Incubation with the antisera for thirty minutes at 37° C is followed by three washings in PBS of ten minutes each. The sections are then mounted in a drop of buffered glycerol and viewed with the fluorescence microscope.

Many different epithelial substrates have been used for indirect immunofluorescence, however skin from the scalp, face, sun-damaged sites and neonatal prepuce has been shown to yield a high rate of false-negative results.[5] Guinea pig lip and oesophagus are also unsatisfactory for standard IIF due to a high rate of false-negatives.[6] In our laboratory, we find the best substrate to be normal human skin from non-sun exposed sites on the trunk and the flexor surfaces of the limbs. This may be obtained from breast reductions, abdominoplasties and other cosmetic procedures. The skin is transproted to the laboratory in normal saline, sectioned and then snap-frozen in OTC compound with the same technique described earlier for skin biopsies. However, certain animal epithelial substrates may have special applications for the specific diagnosis of paraneoplastic pemphigus[7] and bullous pemphigoid.[8]

Every series of indirect immunofluorescence studies must include known positive and negative controls. For the negative controls sections are incubated with normal human serum and with FITC-conjugated anti-human IgG. The later may

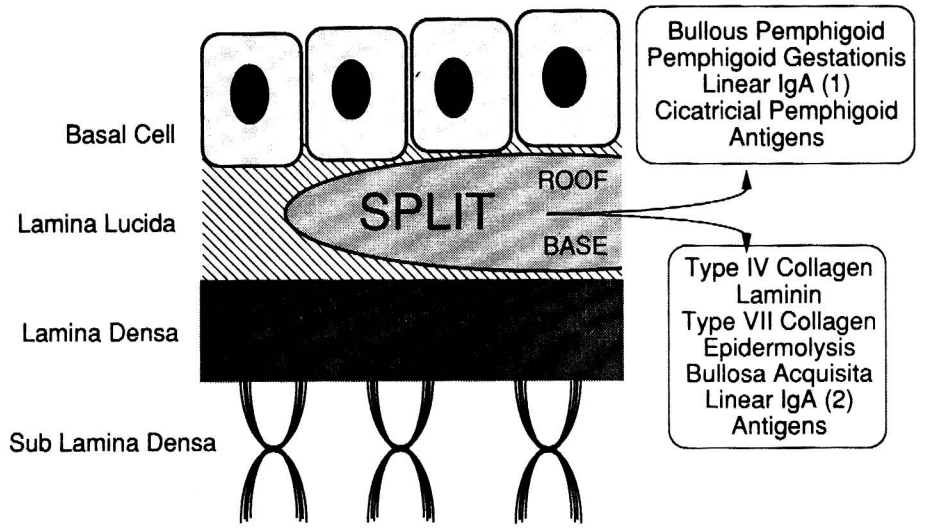

Fig. 2 Split skin localization of BMZ antigens

demonstrate non-specific and undesired specific binding of the conjugate.

The class or subclass of immunoglobulins, the pattern and the site of deposition are noted. Circulating antibodies are most commonly IgG, IgA is present in certain disorders while immunoglobulins of other classes are less common. Immunoglobulins may bind in a linear or discontinous manner either along the BMZ or in the ICS. The patterns of autoantibody binding obtained in the various bullous disorders are shown in Table III.

III Split Skin Techniques

The split-skin techniques have applications in the diagnosis of the immunobullous disorders with separation at the dermal-epidermal junction. Various methods exist to split skin through the lamina lucida, this being the area of least resistance in the basement membrane zone. These methods include suction blister creation or incubation in sodium chloride, trypsin and PBS. Cleavage through the lamina lucida places the hemidesmosomes and upper lamina lucida in the roof of the split and the lower lamina lucida and the sub-lamina densa in the floor (Fig. 2).

The incubation of skin in 1M normal saline for 72 hours at 4^0C more reliably induces cleavage through the lamina lucida

Tabel III: Patterns of autoantibody binding

A Anti-Intercellular Substance	
IgG	Pemphigus vulgaris (PV)
	Pemphigus foliaceus (PF)
	Pemphigus erythematosus (PE)
	Paraneoplastic pemphigus (PNP)
IgA	IgA pemphigus (IAP)
B Anti-Basement membrane zone	
IgG	Bullous pemphigoid (BP)
	Pemphigoid gestationis (PG)
	Epidermolysis bullosa acquisita (EBA)
	Bullous systemic lupus erythematosus (BSLE)
	(Cicatricial pemphigoid (CP))
IgA	Linear IgA disease (LAD)

than the other splitting techniques.[9,10] This technique may be used with biopsies of the patient's skin for direct immunofluorescence or skin substrate for indirect immunofluorescence.

Salt-splitting the patient's skin prior to direct immunofluorescence localizes basement membrane zone immunoreactants to the roof, floor or both locations. This has applications in distinguishing between the subepidermal blistering disorders.[11] The roof or epidermal pattern is found when binding of immunoreactants is to hemidesmosomal and upper lamina lucida antigens. Immunoreactants in the lower lamina lucida and sublamina densa produce a floor or dermal pattern.[12] The split skin technique also increases the sensitivity of detection of BMZ immunoreactants.[12,13]

The cleavage of normal skin substrate prior to indirect immunofluorescence techniques should be performed with added 1mM phenyl methyl sulphonyl fluoride (PMSF). This proteolytic enzyme inhibitor will ensure preservation of antigenic structures in the substrate.[14] The demonstration of whether sera bind to the roof or floor of the split substrate aids in the differentiation of the subepidermal blistering diseases. A roof pattern indicates binding of sera to hemidesmosomal and upper lamina lucida antigens while a floor pattern indicates binding to lower lamina lucida and sub-lamina densa antigens.[15] This technique also increases the sensitivity of indirect immunofluorescence; both the frequency of detection of anti-basement membrane autoantibodies and their titres are increased.[13,16-18]

IV Calcium Enhancement Indirect Technique

The sensitivity of indirect immunofluorescence in pemphigus vulgaris and foliaceus may be increased by tris-acetate buffered saline (TAS) with added calcium chloride.[19] In this technique, TAS with 5mM $CaCl_2$ is used to dilute patients' sera. The mechanism of this heightened sensitivity is unknown. It may occur through stabilization of calcium sensitive epitopes in the target antigens or associated proteins; facilitation of antibody binding; or protecting antigens from proteolysis.[19,20] This technique will not enhance detection of antibasement zone antibodies in bullous pemphigoid or epidermolysis bullosa acquisita.[19]

V Complement-Binding Indirect Method

This three-step indirect immunofluorescence procedure assesses whether circulating autoantibodies are capable of fixing complement. If present, the fluorescent staining of these complement fixing antibodies is also enhanced beyond that achieved by conventional immunofluorescence methods. The enhancement is due to the amplification achieved through binding of more than one molecule of complement to each immunoglobulin and the subsequent visualization of the multiple molecules of complement. The main use of this method is in the diagnosis of pemphigoid (herpes) gestationis in which the autoantibodies avidly fix complement but are difficult to detect using conventional indirect techniques. This technique significantly increases the sensitivity of indirect immunofluorescence in pemphigoid gestationis.[21]

Frozen tissue sections of normal skin are prepared in the standard manner on cover slides. The patient's serum is diluted to 1:2 or 1:4 in PBS and incubated with the sections for thirty minutes at 37° C. Washing with PBS is carried out three times for ten minutes each in the usual fashion, the section are fan-dried and incubated with a source of active complement for thirty minutes at 37° C. The source of

complement is fresh human serum diluted to 1:20 in complement diluting buffer. Sections are washed with PBS, fan-dried and covered with the optimal dilution of fluorescein conjugated anti-C3 for thirty minutes at 37° C. PBS is used to wash the sections once again, they are fan-dried, mounted in buffered glycerol and examined with the fluorescence microscope.

Appendix

One : Michel's Medium

A : Potassium citrate buffer (1M) : To 82.3 ml of disodium hydrogen phosphate solution (14.2 g/ml/ of distilled water) add approximately 17.3 ml of citric acid (21.0 g/ 100 ml distilled water) drop by drop untill pH is 7.0.

B : Buffer : Mix 2.5 ml 1 M potassium citrate buffer, 5.0 ml 0.1 M magnesium sulphate, 5.0 ml 0.1 M N-ethylmaleimide, 87.5 ml distilled water.

C : Michel's fixative : 1. Mix 100 ml buffer, 55 g ammonium sulphate. 2. Adjust pH to 7.0

Two : Phosphate Buffered Saline (PBS)

1. Mix 20.25 g sodium chloride, 3.2 g disodium hydrogen phosphate, 0.39 g sodium dihydrogen phosphate, 2.5L distilled water

2. Adjust pH to 7.4

Three : Tissue freezing

1. Wash tissue in PBS.

2. Place a drop of OCT on cork and immerse in a cold hexane bath.

3. Place tissue biopsy in central OCT, orientate correctly and cover with further OCT.

4. Replace in cold bath until frozen.

5. Store in liquid nitrogen.

Four : Buffered Glycerol Mounting Medium

1. Mix 10 mg p-phenylenediamine, 10 ml distilled water.

2. Add and mix 0.016 g disodium hydrogen carbonate, 0.072 g sodium hydrogen carbonate.

3. Add 90 ml glycerol.

4. Adjust pH to between 8.0 and 9.0.

Five : Direct Immunofluorescence

1. Cut 10 tissue sections of 5 μm on cryotome and place two on each coverslip and fan dry.

2. Wash in PBS for 10 minutes at pH 7.4 and fan dry.

3. Incubate with optimal concentration of FITC-labelled antisera for 30 minutes at 37°C.

4. Repeat 2.

5. Mount in a drop of buffered glycerol and view with fluorescence microscope.

Six : Indirect Immunofluorescence

1. Cut 5 μm sections of substrate.

2. Wash in PBS for 10 minutes and fan dry.

3. Wash in PBS for 10 minutes.

4. Dilute serum to 1:10 or 1:80 in PBS.

5. Incubate serum and sections for 30 minutes at 37°C.

6. Wash with PBS three times of ten minuets each.

7. Incubate with optimal concentration of FITC- labelled anti-immunoglobulins for 30 minutes at 37°C.

8. Repeat 6.

9. Mount in a drop of buffered glycerol and view with fluorescence microscope.

Seven : Split Skin Technique

1. Wash in PBS for 10 minutes.

2. Incubate in 1M NaCl for 72 hours at 4°C.

3. Add 1 mM phenyl sulphonyl fluoride (PMSF) to incubation medium for indirect technique.

Eight : Complement Diluting Buffer

Mix 0.56 g barbitone

8.5 g sodium chloride

0.17 g magnesium chloride

0.03 g calcium chloride

0.19 g barbitone soluble

1.0L distilled water

Nine : Complement Binding Technique

1. Prepare substrate section as per 1-3 of Appendix 6.

2. Dilute patient serum to 1:2 or 1:4 in PBS.

3. Incubate serum and sections for 30 minutes at 37°C.

4. Wash with PBS three times for ten minutes each and fan dry.

5. Incubate sections with 1:5 human serum in complement diluting buffer for 30 minutes at 37°C.

6. Repeat 4.

7. Incubate with optimal concentration of FITC-labelled anti-C3 for 30 minutes at 37°C.

8. Repeat 4.

9. Mount in a drop of buffered glycerol and view with fluorescence microscope.

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