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Review Article

Immunofluorescence in dermatology: A brief review

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ABSTRACT

Immunofluorescence (IF) has been in use for the past five decades, both to investigate the pathophysiology of skin disorders and to help the dermatologists in the diagnosis of various bullous and connective tissue diseases. This review article, deals with different methods, applications, and recent advances in the IF methods used in dermatopathology. Here, we also discuss about the practical aspects of this technique such as handling of skin biopsy specimens and interpretation of direct and indirect IF findings.

Keywords: Immunofluorescence, Direct immunofluorescence, Indirect immunofluorescence, Bullous disease, Connective tissue disease

INTRODUCTION

Immunofluorescence (IF) is a simple, reliable and reproducible technique in immunopathology. It detects the *in situ* and circulating immune deposits involved in the pathogenesis of various diseases, especially those affecting the renal system and skin. The beginning of direct IF (DIF) dates back to 1942, when Coons *et al*. showed the labeling of anti-pneumococcal antibodies with fluorescein in the pulmonary tissue.[1] IF was introduced in dermatology in the 1960s, when Beutner and Jordon revealed the tissue and circulating antibodies in pemphigus vulgaris (PV), pemphigus foliaceus (PF), and bullous pemphigoid (BP).[2-4] IF studies have now become an invaluable supplement to clinical and histopathological examination in a variety of dermatological diseases and are widely used both in research and clinical diagnostics.

In IF, the antibodies that are chemically conjugated with fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate bind directly or indirectly to the antigen of interest in cells or tissue sections. Then, it is visualized by fluorescence or confocal microscopy and quantified by a flow cytometer, an array scanner or an automated imaging instrument. In routine practice, the interpretation of IF study on tissue sections and serum samples is done by fluorescence microscopy.[5,6] There are mainly two types of IF analysis –DIF and indirect IF (IIF).

SPECIMENS FOR IF

The most commonly used specimens for IF studies are: [5-7]

1. Skin biopsy (1.3–6 mm punch biopsies of skin for DIF and 3 mm shave biopsy for IF antigen mapping, which is a type of IIF).
2. Outer root sheath of anagen hair: When patient is not willing for a biopsy (useful in PV).

3. Intact buccal mucosa: PV with isolated mucosal lesions (DIF).
4. Serum: 3 ml of blood without anticoagulant (for IIF)
5. Smears: Sputum, bronchoalveolar lavage, and tissue smears like Tzanck smear (DIF)

INDICATIONS FOR IF IN DERMATOLOGY

1. To assist in the diagnosis and classification of various autoimmune blistering diseases (AIBDs) that may have a confusingly similar clinical profile.
2. In evaluating the prognosis of AIBDs like PV: The intensity of DIF coincides fairly well with the clinical activity.[5]
3. IIF can detect various circulating auto antibodies, which provides an indirect measure of the activity of the disease and helps to decide the most beneficial therapeutic option for the patient.
4. IF antigen mapping (a type of IIF) marks for a particular protein, the presence or absence of which determines the disease classification in hereditary EB (epidermolysis bullosa).
5. The clinical picture of some infections (infections due to herpes simplex virus, and Chlamydia) is often muddled and unclear in patients who are immunosuppressed. In such cases, DIF helps in confirming the diagnosis.[5]

TECHNICAL CONSIDERATIONS

1. There is a necessity of fast reporting and documentation of findings using a digital camera as the fluorescence staining quenches rapidly on exposure to light, more so under the ultraviolet (UV) light of the fluorescence microscope.
2. Three distinct forms of fluorescence (specific and nonspecific fluorescence and autofluorescence) should be considered while reading the IF assay especially with IIF.[5]

Specific fluorescence - Due to a reaction between the substrate and the protein labeled with fluorochrome (antigen-antibody reaction).[5]

Nonspecific fluorescence - Due to coloration of tissues by free fluorescent dye or fluorescent proteins or both.[5]

Autofluorescence - Due to the natural fluorescence of tissues (yellow, blue) when exposed to UV light.[5]

DIF

It is a single step procedure. DIF detects and localizes the immunoreactants deposited in vivo in the patient's skin or mucosa. The primary antibody is labeled with a fluorescent dye. The results are analyzed with respect to the site, number, and the intensity of immune deposits. When multiple immune deposits are identified, the most intense deposit is noted. The sites of deposition (primary and other sites), are identified. Analysis of DIF staining of a biopsy specimen is called "immunohistologic study."[5]

INDICATIONS FOR DIF

DIF is a useful diagnostic tool in many dermatoses [Table 1].[5,6]

It is important to collect DIF specimen from the appropriate site(s). The biopsy should be taken from a fresh blister/lesion, not older than 24–48 hours, for histopathology analysis in AIBDs. A biopsy of normal appearing uninvolved skin within 1 cm of a fresh blister is recommended for DIF in blistering conditions. In PV with isolated mucosal lesions, intact mucosal biopsy is preferred.[5]

Biopsy of a fresh lesion is recommended for DIF microscopy

Table 1: Significance of DIF microscopy in dermatology.

DIF is of diagnostic value
(A) Bullous diseases
Pemphigus (all forms)
Pemphigoid (all forms)
Herpes gestationis/gestational pemphigoid
Dermatitis herpetiformis
Linear IgA bullous dermatosis
Epidermolysis bullosa acquisita
(B) Connective tissue diseases
Discoid lupus erythematosus
Systemic lupus erythematosus
DIF is highly characteristic and of some diagnostic value
(A) Vascular diseases
Allergic vasculitis
Henoch–Schonlein purpura
Essential mixed cryoglobulinemia
Polyarteritis nodosa
(B) Other diseases
Porphyria cutanea tarda and other forms of porphyria
Lichen planus
DIF is not diagnostic but only suggestive of immunologic pathogenesis
(A) Connective tissue diseases
Mixed connective tissue diseases
Systemic sclerosis
Dermatomyositis
(B) Inflammatory dermatosis
Psoriasis
DIF negative bullous disorders
Subcorneal pustular dermatosis
Transient acantholytic dermatosis
Familial benign chronic pemphigus
Acropustulosis of infancy
Dermatopathies due to external energy (friction blistser, electric burns, etc.)

DIF: Direct immunofluorescence

in connective tissue diseases and vasculitis. The appropriate sites of skin biopsy for DIF microscopy in various dermatological conditions are given in Table 2.[5-7]

DIF can also be performed on formalin-fixed paraffin-embedded tissue, which is called paraffin IF. This is done after antigen retrieval with proteases. This is less sensitive than DIF performed on a fresh tissue sample, with sensitivity varying from 0% to 79% in different diseases.[8,9]

TRANSPORT AND STORAGE OF BIOPSY SPECIMEN

Specimens for DIF should not be placed in formalin, as this alters the proteins and significantly diminishes the accuracy of results. The specimen should be transported to the laboratory as soon as possible. The biopsy specimens can be transported on a saline-soaked gauze, in saline solution or in special media (e.g., Michel's or Zeus media). The specimens may also be transported frozen in liquid nitrogen (the specimen should not be allowed to thaw).[5]

Michel's medium contains proteolytic enzyme inhibitors that prevent the autolysis of tissue structures and immunoreactants. The biopsies should be washed in distilled water or normal saline and placed in Michel's medium to ensure the preservation of immunoreactants. Michel's medium preserves immunoreactants for up to 6 months at ambient temperatures.[5,7,10]

Normal saline solution (0.9% NaCl) without addition of calcium or magnesium can also be used for transportation of biopsy specimens.[5,11] Tissue specimens can be kept in 5 ml saline solution for 24–48 hours at room temperature.[5,11] Each laboratory has its own protocols. From our experience, specimens in saline may yield superior sensitivity over other media if processed within 24–48 hours.

Table 2: Ideal site of skin biopsy for DIF microscopy.

Diseases	Types of specimen
Pemphigus (all forms) Pemphigoid (all forms) Epidermolysis bullosa acquisita (EBA) IgA bullous dermatosis Chronic bulllous disease of childhood	Peri-lesional skin within 1 cm of a bulla or intact mucosa (avoid erosions, ulcers, and large bullae)
Dermatitis herpetiformis	Peri-lesional skin within 1 cm of a bulla or normal skin
Lupus erythematosus and other collagen vascular diseases	Involved areas of skin (e.g., an erythematous or active border). Involved, sun exposed skin, and uninvolved, non-sun exposed skin in systemic lupus erythematosus. (Avoid ulcers and old lesions)
Vasculitis	Erythematous or active border of a new lesion (<24 hours old), avoid ulcers and old lesions.
Porphyria cutanea tarda	Involved skin (avoid ulcers and old lesions).

DIF: Direct immunofluorescence

HOW TO REPORT A FLUORESCENT STAINED SKIN BIOPSY SPECIMEN

The prepared IF slides are examined by an experienced pathologist to identify the classes of immunoglobulin (Ig) or other immune deposits, the primary sites of immune deposition, and the patterns of deposition [Table 3]. It should be described under the following headings.[5-8]

Type of immunoreactant

IgG, IgA, IgM, C3, fibrin, C5b-9.

Location of immune deposits

Staining patterns can be classed into five groups

- Intercellular surface staining pattern [Figures 1 and 2].
- Linear basement membrane zone (BMZ) pattern [Figure 3].
- Granular BMZ pattern [Figures 4 and 5]: Granular papillary dermal staining of IgA is diagnostic of dermatitis herpetiformis as shown in Figure 4.
- Shaggy BMZ pattern.
- Vascular or other patterns [Figure 6].

Extent of staining

Focal or diffuse, homogenous, or non-homogenous.

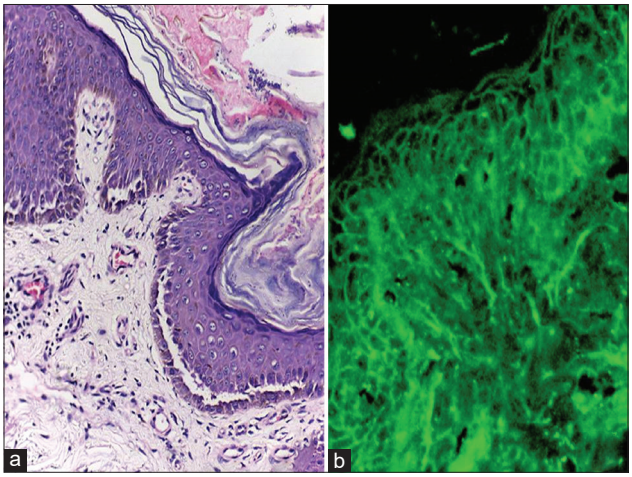

Figure 1: (a): A case of Pemphigus vulgaris, skin biopsy showing suprabasal bulla with basal layer of epithelial cells in "row of tomb stone" arrangement (H and E, 100×); (b): Direct immunofluorescence showing IgG reactive granular (+++) deposits at the intercellular spaces, in a fish net pattern in the epidermis (200×).

Table 3: DIF findings in dermatoses.

Diseases	Pattern and nature of immunoreactants
Pemphigus	Epidermal cell surface deposits of IgG and C3
IgA Pemphigus	Epidermal cell surface deposits of IgA (upper epidermal layers in subcorneal pustular dermatoses type and throughout the epidermis or restricted to lower epidermis in intra-epidermal neutrophilic type)
Pemphigus erythematosus and paraneoplastic pemphigus	Epidermal cell surface and DEJ, homogenous or non homogenous deposits of IgG and C3.
Bullous pemphigoid	Linear homogenous deposits of IgG (epidermal side on salt –split skin) and C3 at DEJ
Linear IgA dermatosis	Linear homogenous deposits of IgA at DEJ
Pemphigoid gestationis	Linear homogenous deposits of C3 at DEJ, rarely IgG
Epidermolysis bullosa acquisita	Linear homogenous deposits of IgG (dermal side on salt – split skin) and C3 at DEJ
Mucous membrane pemphigoid	Linear homogenous deposits of IgG and C3 at DEJ
Dermatitis herpetiformis	Focal granular deposits of IgA at papillary dermis
Bullous eruption of lupus erythematosus	Linear homogenous or non-homogeneous (lupus band), deposits of multiple immunoglobulins, C3 and fibrin at DEJ
Anti-p200 pemphigoid	Linear deposition of IgG and C3 along the DEJ
Lichen planus	Ragged or shaggy fibrinogen deposits at DEJ. Mostly IgM and rarely other deposits on colloid bodies in upper dermis
Lichen planus pemphigoides	Linear IgG and C3 deposits along the BMZ
Porphyrias-(porphyria cutanea tarda, pseudo porphyria cutanea tarda, erythropoietic protoporphyria)	Homogenous deposits of IgG, IgA and less frequently C3 at DEJ and within superficial blood vessels
Cutaneous small vessel vasculitis	Most commonly C3 and fibrinogen deposits within the walls of post capillary venules in the superficial dermis
Henoch- Schonlein purpura	Granular deposits of IgA on the vessel walls in the superficial dermis with or without other immune reactants
Psoriasis	Bright continuous bands of granular positivity along the DEJ with IgG, IgM, C3, and fibrinogen
Mucocutaneous infections	Cytoplasmic staining in HSV type 1-infected cells, nucleolar staining in HSV type 2-infected cells, elementary bodies in Chlamydia trachomatis infected samples and clusters of cysts in Pneumocystis jirovecii infected samples

DIF: Direct immunofluorescence, DEJ: dermoepidermal junction, CSVV: Cutaneous small vessel vasculitis, HSV: Herpes simplex virus, BMZ: Basement membrane zone

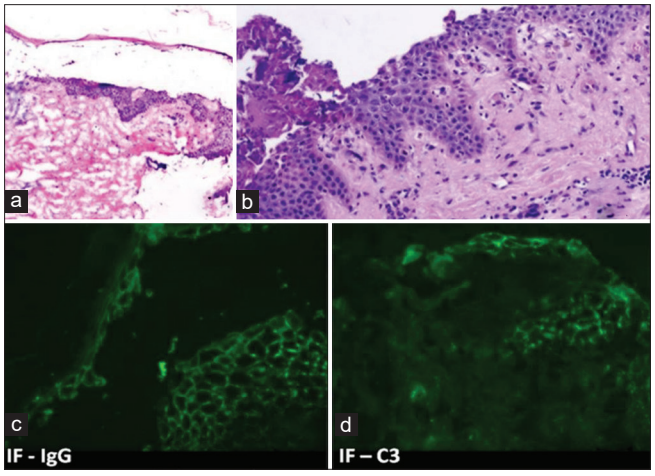

Figure 2: A case of Pemphigus foliaceous (a): skin biopsy showing subcorneal bulla containing acantholytic cells (H and E, 100×); (b): subcorneal bulla containing acantholytic cells (H and E, 200×); (c): direct immunofluorescence showing IgG reactive granular (+++) deposits at the intercellular spaces (intense in the superficial part) in a fish net pattern in the epidermis (200×); (d): C3 reactive granular (++) deposits at the intercellular spaces (intense in the superficial part) in a fish net pattern in the epidermis (200×).

Intensity of staining

Semi-quantitative grading of strength of fluorescence: + to ++++.

Pattern of immune complex deposits

Granular (coarse granules, speckles, threads, and fibrils) or linear.

SERRATION PATTERN ANALYSIS

Studying the serration pattern of BMZ deposits by DIF helps to distinguish between BP and EBA [Table 4]. It requires thinner frozen sections and a higher magnification (more than or equal to 600 fold higher magnification). Hence, it is very difficult to carry out serration pattern analysis in routine laboratory set up.[12,13]

PITFALLS OF DIF

Both false-negative and false-positive results can be observed in DIF microscopy.[5,6]

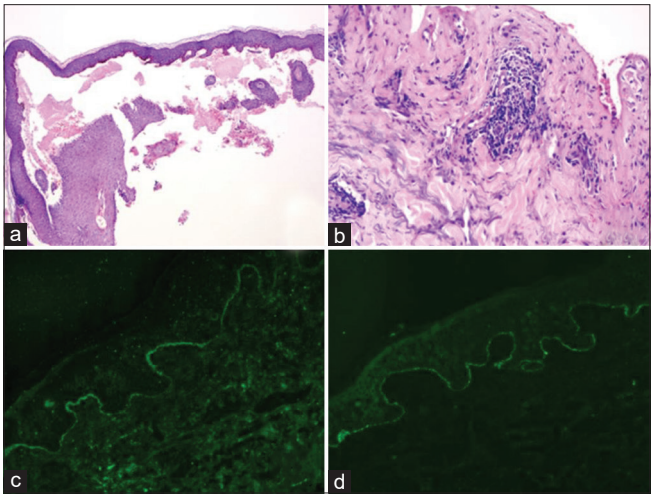

Figure 3: (a): A case of bullous pemphigoid: Skin biopsy showing subepidermal bulla (H and E, 200×); (b): bulla with fibrinous material admixed with moderate amounts of neutrophils and occasional eosinophils (H and E, 400×); (c): direct immunofluorescence (DIF) showing IgG positive reactants - 3+ linear positivity along the basement membrane zone (400×); (d): DIF showing C3 positive reactants - 3+ linear positivity along the basement membrane zone (400×).

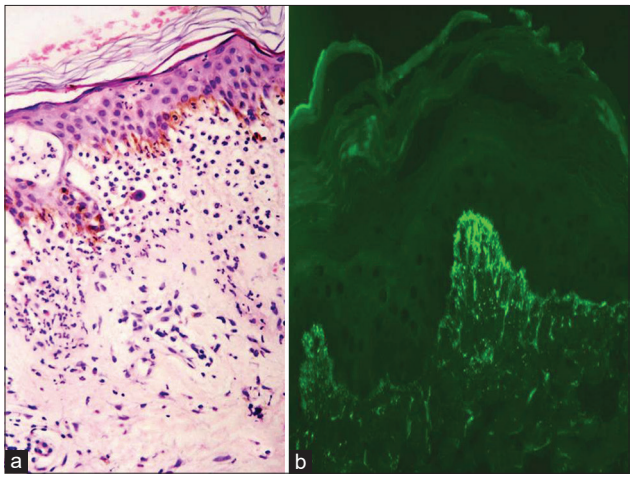

Figure 4: (a): A case of dermatitis herpetiformis - skin biopsy showing sub-epidermal bulla with neutrophilic microabscess and superficial dermal infiltrate of neutrophils (H and E, 100×); (b): direct immunofluorescence showing IgA positive reactants - 3+ at the tip of dermal papilla (200×).

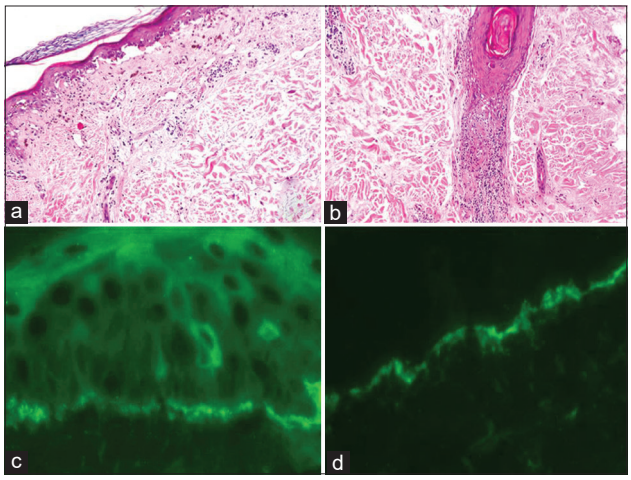

Figure 5: (a): A case of discoid lupus erythematosus - epidermis showing hyperkeratosis, atrophy and vacuolization basal cells (H and E, 100×); (b): showing area with pigment incontinence in superficial dermis and lymphocytic infiltrate around the adnexa and vessels (H and E, 100×); (c): direct immunofluorescence (DIF) showing IgG positive reactive granular (+++) deposits at the dermoepidermal junction (400×); (d): DIF C3 positive reactive granular (+++) deposits at the dermoepidermal junction (400×).

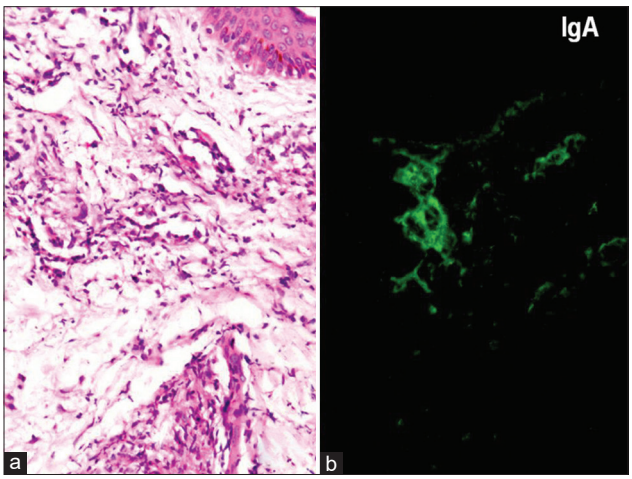

Figure 6: (a): A case of Henoch Schonlein purpura - skin biopsy showing leukocytoclastic vasculitis (H and E, 200×); (b): direct immunofluorescence showing IgA positive reactants in the superficial dermal vessels (400×).

False-negative reactions mostly occur due to following reasons: [5]

- Technical reasons such as formalin contamination of specimen, inappropriate transport medium, or a delay in shipping the sample to the laboratory.
- The specimen may get dried up due to leakage of the fixative solution.
- It is important to keep the biopsy sample in a moist environment because the immunoreactants in dried up biopsies will undergo degradation and may give a false negative result.

False-positive reactions mostly occur due to non-specific staining patterns. Cause for this include: [5-7]

- Crushing and freezing artifacts can produce pemphigus-like pattern.[7]
- Nonspecific granular BMZ staining is observed in conditions such as bullous mastocytosis (with IgM) and elastosis perforans serpiginosa (with IgG) due to intense

Table 4: Serration pattern analysis on DIF microscopy.

Blistering diseases	Serration pattern
• Epidermolysis bullosa acquisita • Bullous Pemphigoid, anti-p200 pemphigoid, anti-laminin-332 mucous membrane pemphigoid	"u- serrated" pattern "n-serrated" pattern

DIF: Direct immunofluorescence

inflammation along the BMZ or due to nonspecific binding of antibodies to altered elastic fibers.

- Biopsy from the sun-exposed skin may show granular IgM staining along the BMZ resembling lupus band.[5]
- Biopsy from the lower leg near the ankle, sometimes exhibits staining around the blood vessel wall, with fibrinogen. Hence, it is confused with vasculitis.[5] Hence, in suspected cases of vasculitis, it is ideal to take a biopsy (for DIF) from the lesion on the most proximal part of the lower limb.[5,14]

The processing of specimens for DIF microscopy can be carried out only in advanced laboratories having facilities such as cryostat for cutting frozen sections, deep freezers (−20°C/−80°C) to store these sections, and a fluorescence microscope to report the findings.[5,14]

IIF

IIF is a semi-quantitative method in which double immunolabeling is used. A secondary antibody labeled with fluorochrome is used to recognize a primary antibody. Here, a normal whole tissue (usually monkey esophagus) is used as the substrate. The patient's serum is layered on the substrate and then fluoresceinated antibodies are applied. The method detects the circulating antibodies in the serum. IIF is 10–15 times more sensitive than DIF. IIF titers may correlate with the disease severity. So in addition to diagnosis, IIF has prognostic value as well and is useful to monitor the response to therapy.[5]

The substrate is first incubated with a series of dilutions of patient's serum in phosphate buffer solution for 30 minutes. Unlabeled primary antibodies from the patient serum are allowed to bind to the target molecule in pre-prepared tissue samples such as monkey esophagus or normal human skin. If circulating auto antibodies are present in the serum, it will bind to the respective antigens in the substrates and are detected by incubation with appropriate fluorescent (usually FITC) labeled, mouse anti-human IgG, and/or IgA. Screening for antinuclear antibody (ANA) should be done at dilutions of 1/40, as at lower dilutions, false-positive results can occur.[5]

INDICATIONS FOR IIF

Diagnosis of autoimmune bullous diseases

IIF is used to demonstrate the antibodies in the patient's serum directed against specific antigens in the following diseases.[5]

- PV
- PF
- Paraneoplastic pemphigus
- Linear IgA bullous dermatosis
- Chronic bullous disease of childhood
- Dermatitis herpetiformis
- BP
- Pemphigoid gestationis
- Mucous membrane pemphigoid
- EBA
- Bullous systemic lupus erythematosus (bullous SLE)

Diagnosis and assessment of disease activity in various autoimmune diseases

IIF is used to demonstrate ANAs in connective tissue diseases such as SLE, mixed connective tissue disease, and systemic sclerosis and to demonstrate antineutrophil cytoplasmic antibodies (ANCA) in ANCA associated small vessel vasculitis (AVV).[5,15-18]

The following precautions can yield an accurate result in IIF: [5]

1. Adequate washing during each step of treatment with the antibody and fluorescent conjugate is very important to remove the unwanted immunodeposits.
2. Appropriate storage of the tissue sections at adequate temperature (−70 °C to −80°C)
3. Patient's sera should be refrigerated until tests are performed. Repeated freezing and thawing should be avoided since this can lead to rapid loss of activity of the antibody.[5]
4. It is important to compare the relative intensities between the positive and background staining in control slides while interpreting the IIF.[5]

INTERPRETATION OF ANA TEST

ANA test is a standard screening assay for antibodies produced in autoimmune or ANA associated rheumatic disease and ANCA associated vasculitis.[15-17]

Hep-2 IIF is the method recommended by the American College of Rheumatology for ANA screening.[15,16] More than 100 autoantibodies to different nuclear and cytoplasmic antigens can be identified by this technique.[15-19] The nuclear patterns and the titer are the important aspects to be considered while interpreting IIF for ANA.

Patterns of ANA commonly reported by most laboratories are homogeneous, speckled, nucleolar, centromere, and proliferating cell nuclear antigen (PCNA).[15] The ANA patterns associated with specific diseases are mentioned below.

Homogeneous

SLE, chronic autoimmune hepatitis, and juvenile idiopathic arthritis [Figure 7a].

Nucleolar

Patients with systemic sclerosis and those with clinical manifestations of other systemic autoimmune rheumatic diseases [Figure 7b].

Speckled

In varying degrees in distinct systemic autoimmune rheumatic diseases, in particular, SLE, systemic sclerosis, mixed connective tissue disease, and undifferentiated connective tissue disease [Figure 8a].

Centromere

Limited cutaneous systemic sclerosis [Figure 8b].

PCNA

Earlier, PCNA was considered highly specific for SLE, but this specificity is now debated. Clinically, it is associated with systemic sclerosis, autoimmune myopathy, rheumatoid arthritis, and hepatitis C virus infection [Figure 9].

Interpretation of ANCAs

ANCAs are important laboratory markers to support the diagnosis of ANCA-AAV, including granulomatosis with polyangiitis and microscopic polyangiitis. Conventionally, screening for ANCA by IIF is done and IIF positive samples are further evaluated for antibodies to proteinase 3 (PR3) or myeloperoxidase (MPO) by specific immunoassays.[17]

According to the IIF pattern on ethanol-fixed neutrophils, ANCAs are classified into[17]

- C-ANCA (Poteinase 3 ANCA or PR3 ANCA): Granular cytoplasmic staining with accentuation between the nuclear lobes
- P-ANCA (MPO-ANCA): Perinuclear and/or nuclear staining [Figure 10]
- Atypical ANCA: Diffuse cytoplasmic and "perinuclear" staining. It is also termed as atypical P- ANCA or X-ANCA or snowdrift-ANCA.

The patterns correlate with varying degrees of specificity to specific neutrophil granule contents. ANCAs can be IgG (most frequent), IgM, or IgA.[17]

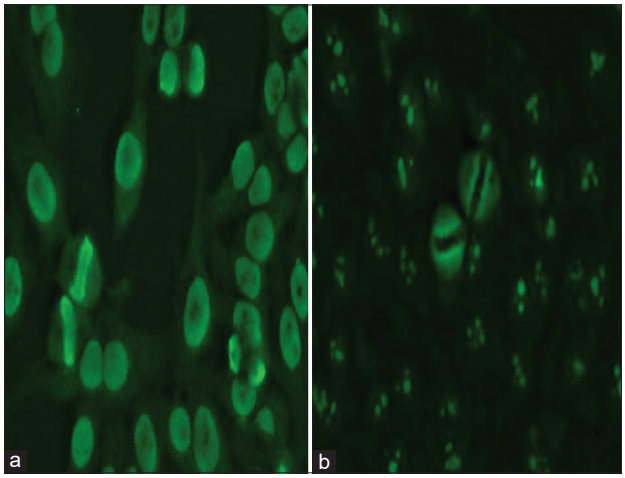

Figure 7: (a): Indirect immunofluorescence photomicrograph of Hep 2 cell lines showing homogenous pattern (400×); (b): Indirect immunoflourescence photomicrograph of Hep 2 cell lines showing nucleolar pattern of nuclear fluorescence (400×).

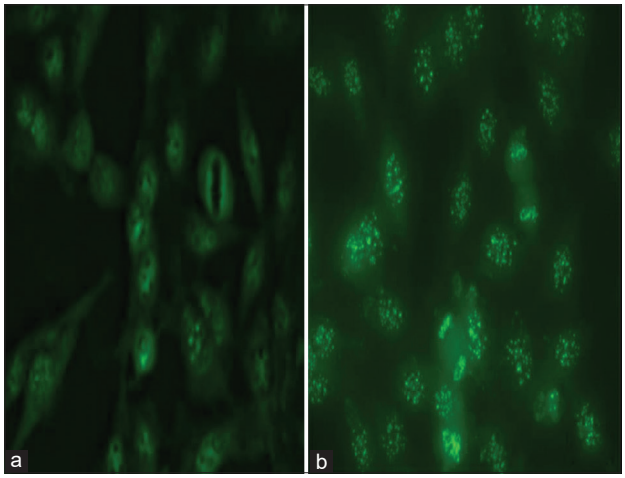

Figure 8: (a): Indirect immunofluorescence (IIF) photomicrograph of Hep 2 cell lines showing speckled pattern of fluorescence (400×); (b): IIF photomicrograph of Hep 2 cell lines showing centromere pattern of fluorescence (400×).

In the revised 2017 international consensus on ANCA testing in small vessel vasculitis, the use of high-quality immunoassays with PR3-MPO-ANCA is recommended as the preferred method (without the categorical need for IIF) in suspected cases of ANCA-associated vasculitides.[17]

OTHER FORMS OF IIF

Antigen mapping or immunomapping

This modified IIF technique was described by Hintner *et al.* in 1981.[5,20] Patient's own skin is used as the substrate. Immunofluorescence antigen mapping (IFM) helps to determine the exact site of cleavage in AIBDs and the abnormalities in the distribution of mutated structural

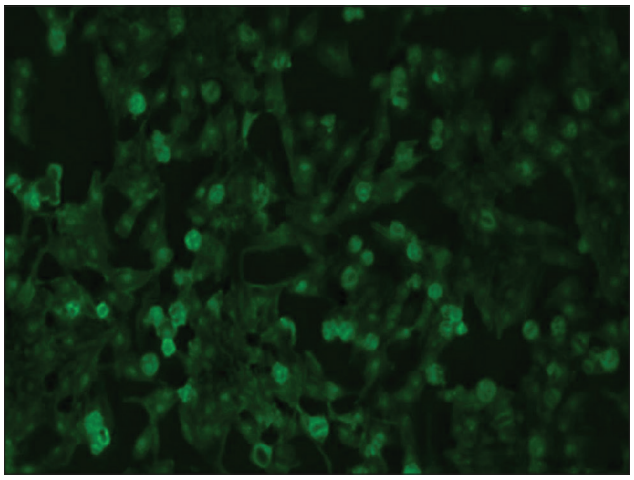

Figure 9: Indirect immunofluorescence photomicrograph of Hep 2 cell lines showing proliferating cell nuclear antigen pattern of nuclear fluorescence (400×).

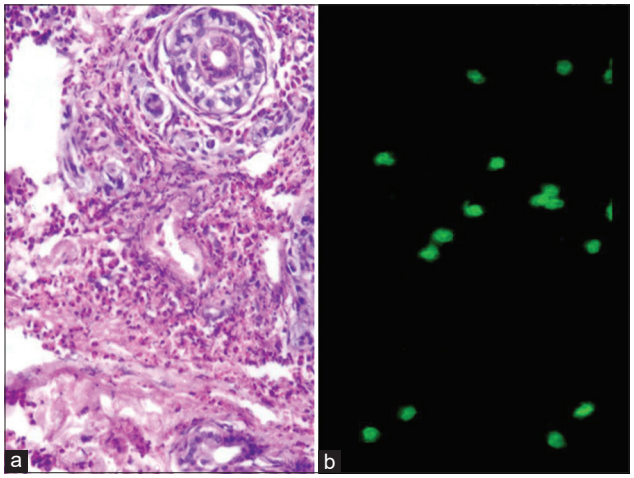

Figure 10: (a): A case of eosinophilic granulomatosis with polyangiitis, section of skin with eosinophilic granuloma and small vessel vasculitis in the dermis (H and E, 200×); (b): Indirect immunofluorescence photomicrograph of ethanol fixed neutrophil preparation showing anti neutrophil cytoplasmic antibody pattern of perinuclear fluorescence (400×).

proteins in hereditary EB. IFM has taken over transmission electron microscopy as the preferred method for preliminary diagnosis of EB.[20]

In IFM, frozen sections of shave biopsy of patient's skin are stained with commercially available monoclonal antibodies (primary antibody) directed against different antigenic components of the BMZ/epidermis such as keratin 5/14, laminin 332, and type VII and 1V collagen.[20] A secondary antibody tagged with fluorochrome detects the immune complex. The staining pattern is then compared with that of normal healthy skin to confirm whether the antigen expression is normal, reduced, or absent.[5,20]

Complement fixation

Here, patient's serum is layered on the substrate, and a source of complement is added. Then, fluoresceinated anti-complement antibodies are used to detect the presence of complement in the tissue. This type of IIF helps to detect small quantities of complement fixing antibodies.[5]

BIOCHIP mosaic slides (Euroimmun, Lubeck, Germany)

BIOCHIP mosaic slides are useful in patients with AIBDs to screen for autoantibodies. The commercially available, ready-to-use slides containing six different substrates (monkey esophagus, primate salt-split skin, recombinant BP180 NC16A, membrane-bound desmoglein (Dsg)1 ectodomain, Dsg3 ectodomain, and the C-terminal globular domain of BP230) in a miniature field are used. To these ready-to-use slides, serum from patients with suspected AIBD is added which is then examined under fluorescence microscope. This method does not require frozen sections of the substrate.[21]

Salt split technique (SST)

It is a procedure that reliably differentiates between various subtypes of immunobullous disorders that are characterized by separation at the dermoepidermal junction (DEJ). It was introduced by Gammon *et al*.[22] There are two types of SST - direct salt split skin test (D-SST) and indirect salt split skin test (I-SST).[5,23]

D-SST is performed on patient's skin biopsy specimen. A fresh skin biopsy from clinically normal appearing "patient" skin can be used for D-SST or alternatively D-SST can be performed on the specimen that was used for routine DIF. Punch biopsy specimens are incubated in 5 ml of NaCl (1 mol/L) at 4°C for 24 hours. The epidermis is then teased from the dermis with the use of a fine forceps. The specimens are then processed in the same manner and treated with IgG and C3 conjugates as in DIF. D-SST aids to determine the site of *in vivo* deposition of bound auto antibodies within the DEJ (whether deposited on the blister roof or on the blister floor or on both sides of the artificial split).[5]

For I-SST, a sample of normal human skin is used as the substrate. After artificially inducing the junctional split, cryocut sections are prepared. Then IIF with patient's serum is carried out. This is more sensitive than routine IIF and is very helpful when a lesional biopsy is not available.[5]

CONCLUSION

IF plays a significant role in the diagnosis of vesiculobullous disorders, connective tissue disorders and vasculitis. It should be interpreted and correlated with clinical features,

other investigation results, and histopathology findings to arrive at the correct diagnosis.

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Conflicts of interest

There are no conflicts of interest.

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