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IMMUNOFLUORESCENCE: TECHNIQUE AND ITS APPLICATIONS TO ORAL DISEASES.

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ABSTRACT

Immunofluorescence (IF) is the labeling of any substrate with fluorescent dyes. It enables detection of antibodies, antigens, complement components and fibrin. It is one of the common laboratory techniques that has wide applications in clinical diagnosis and research. It's a good diagnostic adjunct to many autoimmune mucocutaneous diseases. This article aims to provide a brief review on the types, technique and the applications of immunofluorescence technique in oral diseases.

KEYWORDS: Immunofluorescence, Technique, Antigen, Antibody, Autoimmune diseases.

INTRODUCTION

Immunofluorescence is a histochemical laboratory staining technique used for demonstrating the presence of antibodies bound to antigens in tissues or circulating body fluids. These techniques are essential to supplement clinical findings and histopathology in the diagnosis of immunobullous disorders. They permit early diagnosis, treatment and subsequent monitoring of disease activity in patients with potentially life-threatening disorders.[1,2,3] 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.[2]

Luminous materials were known since the times of the Greeks and Romans. Chinese books were written about fluorescence and phosphorescence as far back as 1500 B.C.[4,5] Then in the 17th century phosphorescent substances were discovered. Many consider Athanasius Kircher as the founder of fluorescent science. Albert Coons and Melvin Kaplan are the inventors of immunofluorescence and in 1942, they showed the labeling of antipneumococcal antibodies with fluorescein in the pulmonary tissue.[4,5,6]

IF was introduced into dermatology in 1960s. In 1963, the granular deposits of IgG and C3 were first described along dermal-epidermal junction in lesions of lupus erythematosus. In 1964, Beutner and Jordon revealed tissue and circulating antibodies in autoimmune vesiculobullous diseases, especially pemphigus vulgaris.[1,4] Since then, this technique has resulted in many advances in cell Biology.

The term "Fluorescence" refers to the process by which atoms absorb photons of one wavelength and emits photons at a longer wavelength. The basic principle of IF is the primary reaction between antibodies chemically combined with fluorescent dyes and cells or tissue fixed antigens that are visualized using a suitable microscope such as fluorescence or confocal microscope or is quantified using a flow cytometer, array scanner or automated imaging instrument.[7]

The most commonly used labelers for this technique are fluorochromes, enzymes, radioactive and electro-opaque compounds. Among the fluorochromes, commonly used ones are fluorescein isothiocyanate (FITC), tetramethyl rhodamine isothiocyanate (TRITC).[4,7] Fluorescein isothiocyanate (FITC) is of green color, with absorption and emission peak wavelengths of 490 nm and 520 nm, respectively and Rhodamine, is of red color, with absorption and emission peak wavelengths 520nm and 610 nm respectively.[4,6,8] Texas red, is of red color, with absorption and emission peak wavelengths 595nm and 615 nm, R-Phycoerythrin (PE) is of orange/red color, with absorption and emission peak wavelengths 565nm and 575nm respectively.[6]

During the IF procedure, the fluorochromes can be attached to antibodies which will then bind to specific chemical structures on or inside cells. The chemical and physical properties

of fluorochromes determine when and where these dyes are useful in various biological assays. For example, the fluorochromes that bind to DNA, can get into living cells, but most DNA -binding fluorochromes cannot get past the cell membrane. Those fluorescent dyes that cannot get past an intact cell membrane, such as propidium iodide (PI), are often used to distinguish live from dead and dying cells.[7]

Types of immunofluorescence

There are mainly two types of IF, Direct IF (DIF) and Indirect IF (IIF), with variants in them.

Direct immunofluorescence (DIF)

In DIF, the antigen in a cell or a tissue is visualized by direct labeling with fluorescent antibody. This technique demonstrates autoantibodies attached to the patient's tissue. It was described initially by Coons.[9,10]

Technique

- Choice of an appropriate site for biopsy, selection of a suitable transportation medium and getting the testing facility at the earliest are the most important prerequisites for the success of an immunofluorescence testing and failure at any of these points results in false negative outcomes.[11]
- Tissue sample is obtained from a clinically unaffected perilesional skin or mucosa that is adjacent to a new vesicle or bullae for bullous diseases and from the lesional site for lichen planus, vasculitis and connective tissue diseases. In collagenosis, recent lesions with less than 60 days are avoided, but in vasculitis, preference is given to recent lesions with upto 24hrs of evolution. In some cases a biopsy can be obtained by inducing a vesicle by rubbing the mucosa before taking the biopsy. Biopsy of the lesional tissue for DIF is not advisable as the immune deposits are degraded by intense inflammation or damage in the basal membrane zone, rendering DIF falsely negative.[2,9] Sample from long standing lesions are not advised as there would have been loss of immunoreactants from the lesion. Biopsies distant from the clinical lesion can also produce significant results.[9]
- 3-5mm punch or surgical biopsy is advisable and punch samples show greater sensitivity than the scalpel biopsies.[1,2,9] It should have an appropriate extension and depth that involves both the epidermis and dermis in sufficient propotion.[4] Siegel has described an intraoral wedge biopsy technique for DIF studies. This technique offers the advantage of

obtaining highly diagnostic, mirror-image soft tissue specimens from a single perilesional site while minimizing postsurgical morbidity to the patient.[12]

- Among oral sites, good sensitivity has been shown for the biopsies obtained from the floor of mouth and hard palate, and least sensitivity with gingival and dorsal side of the tongue. Gingival biopsies are usually not advised, because these samples generally show high degree of unspecific inflammation, and diagnosis with gingival biopsies is technically challenging, as the epithelium is frequently detached from the chorium underneath or is lost during the sampling process, which leads to an incorrect sample preparation or to a wrong interpretation. The gingival biopsy might also leave a periodontal defect.[9]
- Fast freezing of the tissue is extremely important. This is accomplished by immersing the biopsy specimen in liquid nitrogen or cold carbon dioxide or in a hexane bath.[1] The biopsy specimen is then mounted and sectioned in a cryostat. If immediate freezing is not possible, special transporting medium is used.
- Normal saline is sufficient as the transport medium if the specimen is transported with in 24hrs. Specific buffered hypertonic saline solution such as Michel's medium is used if the transport takes greater than 24hrs. This medium is composed of ammonium sulphate, N-ethyl-maleimide and magnesium sulphate in a citrate buffer and allows conservation of the specimen for upto two weeks. Due to presence of proteolytic enzyme inhibitors, it prevents tissue degradation without damaging the immunoreactants.[2,4,11] Adequate pH of 7.0 to 7.2 should be maintained during the transport to reduce variable staining and other tissue artifacts.[6]
- Before the start of the procedure, the holding solution is rinsed off in neutral buffer solution. This removes ammonium salts and any residual blood proteins.[2] Specimen is frozen, embedded, sectioned and placed on slides. Slides are incubated with fluorescein labeled antibodies directed against antigens, complement or fibrinogen and examined under fluorescent microscope.[11] Labeled antibody-antigen complexes shine yellow-green. The contrast can further be increased by staining with Evans red.
- Formalin fixation is unadvisable as it produces increased background staining. It also causes auto-fluorescence of the tissue and IF becomes patchy. The formaldehyde also prevents binding of the dye to the substrate of interest. Aldehyde-induced fluorescence is due to reaction of free aldehydes with tissue components and the effect is compounded when fixation is longer or warmer. This is prevented by addition of methanol along with

formalin. Aldehyde blocking can minimize aldehyde-induced fluorescence by reducing the -CHO groups to -OH with sodium borohydride.[3]

- There are several advantages of this technique, such as reduction in the number of steps in the staining procedure**, s**horter staining times**, s**impler dual and triple labelling procedures**,** avoids antibody cross-reactivity or non-specificity, which can lead to increased background signal.
- The disadvantages include lower signal, generally higher cost, less flexibility and difficulties with the labelling procedure when commercially labelled direct conjugates are unavailable.[7]
- One of the major disadvantage of DIF is the need for fresh frozen tissue (FFT). However, DIF of formalin fixed-paraffin embedded tissue can be done in cases where FFT is unavailable and is reliable for diagnosis purpose. Good results can be obtained with adequate unmasking of antigens. The intensity of staining may be slightly less but the pattern of staining remains the same.[13]

Indirect Immunofluorescence (IIF)

This method involves incubation of a substrate containing a fixed antigen with unlabelled antibody, which becomes associated with the antigen. Fluorescent antibody is added to the substrate & is visualized. This demonstrates autoantibodies in the serum.[10]

Technique

- It is a two step serological procedure and is usually performed after DIF reveals deposition of the antibodies. Blood is drawn from the patient and is centrifuged to obtain the serum, which is refrigerated at about -20^{0}C if arrival time in lab is expected to exceed 2 days.[11]
- Serum is subjected to serial dilutions in phosphate buffer solution and is incubated with a frozen section of epithelial substrate. Monkey or guinea pig esophagus, rodent's bladder, liver & heart and human skin are used as the substrates.[2,11]
- If there are auto-antibodies in serum, they attach to the homologous structures on the substrate. The excess serum is washed off. It is then incubated with fluorescent labelled anti-human antibody. The excess is washed off and the section is examined under fluorescent microscope. Simultaneously, the IIF of positive and negative controls is tested.

The advantages of IIF include greater sensitivity, amplification of the signal, relatively inexpensive, quality controlled, available in array of colors, more time-efficient and the disadvantages include potential cross-reactivity, sometimes may exhibit a high background with endogenous immunoglobulins and the need to find primary antibodies that are not raised in the same species or of different isotypes when performing multiple labeling experiments.[7] In general, the drawbacks of IF techniques include - need for the special microscope and its cost, preference for frozen sections, preparations need refrigeration and preparations cannot be kept for long time.

As with most fluorescence techniques, a significant problem with immunofluorescence is photo bleaching i.e. the quick fading of fluorescence under illumination. Loss of activity caused by photo bleaching can be controlled by reducing the intensity or time-span of light exposure, increasing the concentration of fluorophores and by employing more robust fluorophores that are less prone to bleaching.

Salt- split technique

This is an enhanced method of IF, which is used to determine the specific component of BMZ that are targeted by antibodies. It was developed in 1984. It distinguishes between subepidermal blistering conditions with similar DIF findings. Sensitivity is increased with this technique as dermal and epidermal portions can be studied separately.[4] During the procedure, the human skin is incubated with 1 mol/L of NaCl solution, which results in separation of the two layer at the site of lamina lucida portion of the BMZ exposing the antigens at the basement membrane zone.[4,11] Antibodies binding antigens in the hemidesmosomal and upper lamina lucida (pectin, BP antigen-BP 230) produce roof or epidermal pattern and the antibodies binding antigens in the lower lamina lucida and sublamina densa (laminin, type IV & VII collagens) produce a floor or dermal pattern.[4]

Double Immunofluorescence

Double IF is carried out just as single labeling. Antibodies derived from different animal can be mixed and incubated as a cocktail (example: rabbit anti-A and mouse anti-B). The same is valid for secondary antibodies (example: goat anti-rabbit Texas Red conjugated and goat anti-mouse fluorescein conjugated). If secondary antibodies cross-react, they should be pre absorbed against each other or with liver acetone powder of the appropriate animal. There may be reduction or loss of signal with one of the antibodies, if the antibody binding sites of the primary antibodies are in close proximity.[14,15]

Complement –binding indirect immunofluroscence

It's a three step IIF technique and is more sensitive than the conventional IIF. It assess if circulating autoantibodies are capable of fixing the complement. It is mainly used in situations where in, only few antibodies bind to tissue antigens and hence they cannot be detected by IIF. It is used in cases of pemphigoid gestationis.[1,2]

Lupus band test (LBT)

It is a DIF technique for demonstrating a band of localized immunoglobulins at the dermal-epidermal junction in the patients with lupus erythematosus. It was described initially by Burnham et al, in 1963, who demonstrated a band of immunofluorescence in Lupus erythematosus using DIF and since then this IF technique has been known as the lupus band test. A homogenous solid well demarcated band or stripled band is produced and the antibodies noted are IgG & IgM and occasionally IgA with IgM, C3 & C4.[16]

Quantitative immunofluorescence

In this technique, antigen is bound to a solid phase and is then exposed to a serum sample containing specific antibody. A fluorescein-labeled antibody is added to reveal the antibody that reacts specifically with the immobilized antigen. A fluorometer is used to assay the amount of fluorescence emitted by the second antibody. Since the amount of fluorescent antibody added to the system is fixed, the amount that remains bound is directly proportional to the concentration of the antibody present in the sample. Thus a quantitative correlation can be drawn between the intensity of fluorescence and the concentration of antibody added in the first step. The applications are to study the antigen-antibody reaction patterns in immune-mediated diseases, for diagnosis of syphilis and some viral disease, for the detection of antinuclear antibodies, anti double stranded DNA antibodies and other antibodies.

The sandwich technique

The technique described by Weller and Coons is designed to visualize a specific antibody produced within a cell. Technically, the tissue is first fixed with ethanol to prevent washing away the antibody during the test. Treatment with polysaccharide antigen constitutes the first layer. Antibody in tissues or smears is reacted with umlabelled antigen. After washing, the fluorochrome-labelled antibody is applied. This will react with the antigen and so will demonstrate the site of the original antibody.[8]

Antigenic mapping method

This method is used as an adjunct to electron microscopy to differentiate between the major forms of epidermolysis bullosa. The antigenic components – bullous pemphigoid antigen, laminin and type IV collagen at the dermal-epidermal junction are studied by performing an IIF on a mechanically induced blister. In superficial blisters, all the three antigens are noted on the floor, in dystrophic blisters, they are detected on the roof and in junctional blisters, bullous pemphigoid antigen are seen on the roof and type IV collagen on the floor.[1,2]

Calcium enhanced indirect technique

It is a form of IIF in which the sensitivity of the technique is enhanced by the addition of calcium chloride. It acts through the stabilization of calcium sensitive epitopes in the target antigens or associated proteins, or facilitation of antibody binding, or protecting antigens from proteolysis.[2]

Double staining method

This is used to demonstrate the co-distribution of two antigenic substances in the tissues by conjugating FITC and TRITC. This can be used as direct or indirect method and indirect method has high sensitivity.[1]

Some practical applications of IF in diagnostic pathology are: analysis of antigens in fresh, frozen or fixed tissues, sub-cellular localization of antigens in tissue culture monolayers, observation of bacterial or parasitic specimens, detection and localization of the presence or absence of specific DNA sequences on chromosomes and defining the spatial-temporal patterns of gene expression within cells/tissues.[7] Other applications include immunohistochemistry, immunocytochemistry, fluorescent in situ hybridization, mineralogy, gemology, chemical sensors, fluorescent labeling, dyes, biological detectors, fluorescent lamps, geology, forensics and paintings.

Autoimmune bullous diseases are characterized by inappropriate production of autoantibodies by the patient that are directed against various constituents of the molecular apparatus that hold epithelial cells together or that bind the surface epithelium to the underlying connective tissue. The ensuing damage produced by the interaction of these autoantibodies with the host tissue is seen clinically as a disease process.[2,4,7,11,17] Each disease is characterized by the production of specific types of Autoantibodies. The identification of the antibodies and the tissue against which they are targeted is important

diagnostically in immune mediated diseases. IF technique can be used diagnostically for the identification of autoantibodies in such diseases. Table -1 summarizes the immunofluorescent findings of few of the autoimmune diseases.

Table: 1. Immunofluroscent findings in autoimmune disorders[2,4,9,10,11,17]

Disease	Antigen	Antibodies	Pattern of immunofluroscence
Pemphigus group of lesions	Dsg3, Dsg1, Desmocollin, Plakin family	IgG, IgM, IgA, C3, Fibrin	Intercellular deposits between epidermal cells in network pattern
Paraneoplastic pemphigus	Dsg, plakin family		Intercellular deposits & linear or granular BMZ band
BP	BPAG1,-2	IgG, IgM, C3	Linear deposits along BMZ
MMP	BPAG1,-2, integrin	IgG,C3	Linear deposits along BMZ
Epidermolysis bullosa acquistica	Collagen VII	IgG, C3	Linear deposits along BMZ
Linear IgA disease	LAD, BPAG1,-2	IgA,occasionally C3, Ig G also.	Linear deposits along BMZ
Bullous SLE	Collagen VII	IgG, IgM	Linear deposits along BMZ
Lichen planus		IgG, IgM, IgA, C3, Fibrin.	Deposits in ovoid bodies. Ragged fibrin band along BMZ
vasculitis		IgA, C3, IgG, IgM.	
Porphyrias		IgA, C3, IgG, IgM(rare).	
Dermatitis herpetiformis		IgA, C3, IgG.	

CONCLUSION

As some of the diseases are potentially life threatening, use of a diagnostic test is of critical importance. Immunofluorescence is a useful tool for diagnostic clarification. Immunofluorescence can be specific up to a certain degree and can be used as an auxillary laboratory tool for the diagnosis of the disease under investigation. It can vastly assist both the clinician and the pathologist in arriving at a correct diagnosis and also for monitoring the effects of various therapies involved in treatment of the disease.

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