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Review Article **Oral Pathology and Microbiology**

Immunofluorescence in Immune Mediated Diseases of Oral Cavity

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Abstract

Immunofluorescence is an immunological method used to detect the presence of immune deposits in tissue or serum. This has emerged as a valuable technique in diagnosing vesiculobullous disorders, especially when the histopathology is inconclusive. This review explores the diverse applications of immunofluorescence in oral disease, diagnosing autoimmune conditions like Pemphigus Vulgaris and Mucous Membrane Pemphigoid. This method utilises fluorescent-labelled antibodies to detect specific antigens or proteins in the tissues.

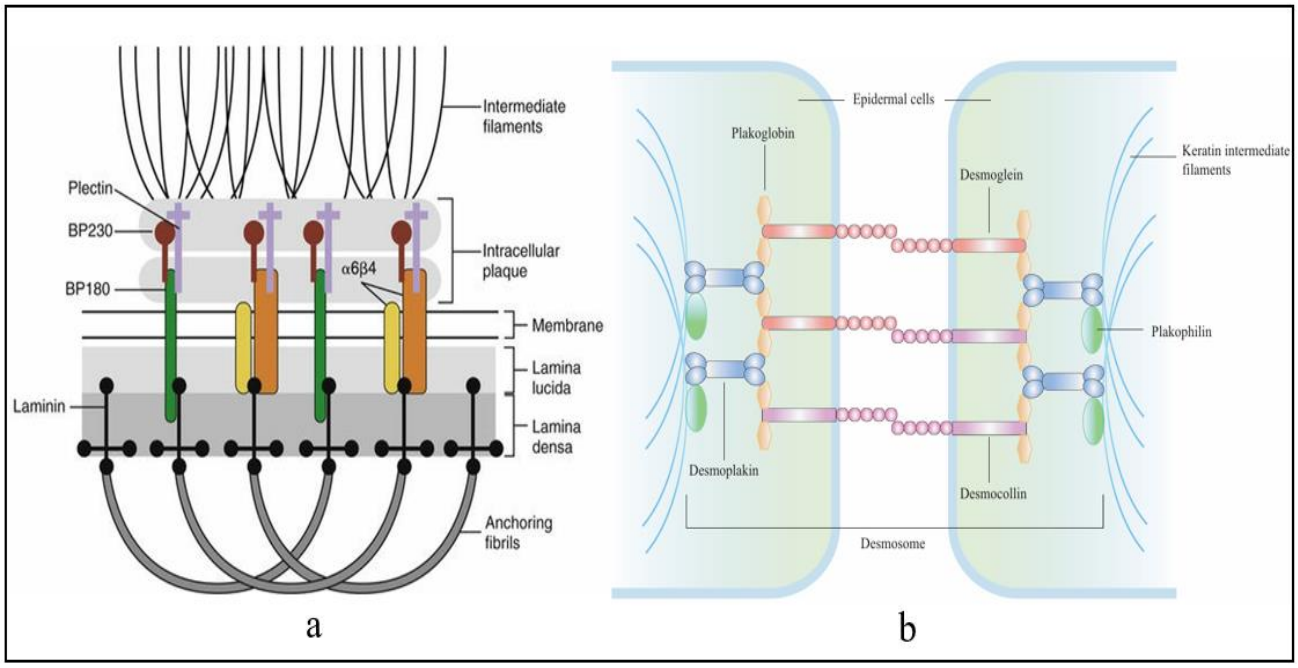
Keywords: Direct immunofluorescence, immunobullous lesions, immunofluorescence, Pemphigus, vesiculobullous lesions.

INTRODUCTION

Immunobullous disorders represent a group of diseases where autoantibodies are directed against key structural components that maintain the integrity of epithelial cells or that hold the surface epithelium to underlying connective tissues [1]. Immunofluorescence (IF) is a laboratory method used to identify antibodies attached to specific antigens in tissue or serum samples. The method traces its origins to the work of Coons and Kaplan [2]. Fluorescence is a phenomenon in which certain materials absorb light of one wavelength and subsequently emit it at a different wavelength. Fluorescence occurs because of the atomic structure of these materials. In atoms, electrons exist in specific orbits around the nucleus, each with a distinct energy level. When an electron absorbs energy from a photon, it moves to a higher energy orbit. However, these higher energy states are unstable and brief, typically lasting less than 10 seconds. Consequently, the electron releases a portion of the absorbed energy as heat, while the remaining energy is emitted as a photon [3]. In IF techniques, antibodies are tagged with fluorescent dyes such as fluorescein isothiocyanate (FITC) or tetramethyl rhodamine isothiocyanate (TRITC) [4]. This histochemical staining method can act as a adjuvant in the diagnosis of immunobullous diseases, aiding in early detection and treatment of these potentially life-threatening conditions [5].

Ultrastructure of Normal Adhesive Junction

Oral mucous membranes consist of an epithelium and an underlying connective tissue. The epithelium is connected to the underlying connective tissue by a complex interaction of various proteins. Hemidesmosome links the epithelium to basal lamina and through some extracellular molecules to rest of the extracellular matrix. It contains transmembrane protein integrin, which bind to laminin and bullous pemphigoid antigen 180 (BP180), cytoplasmic adaptor proteins such as bullous pemphigoid antigen 230 (BP230) and plectin which forms the attachment plaque for intermediate filaments. Adjacent epithelial cells are connected to each other with specialised structures called desmosome. Desmosome contains transmembrane proteins desmocolins and desmoglein and cytoplasmic proteins like desmoplakin, plakoglobin and plakophilins [6, 7].



Schematic diagram of hemidesmosome (a) and desmosome (b)
Courtesy: (a) Antonio nanci Ten Cate's oral histology: Development, structure, and function, Elsevier. 8th edition. (b)Sun S, Zhong B, Li W, Jin X, Yao Y, Wang J, Liu J, Dan H, Chen Q, Zeng X. Immunological methods for the diagnosis of oral mucosal diseases. Br J Dermatol. 2019 Jul;181(1):23-36.

Types and Technique

There are three main types of immunofluorescence techniques.

1. Direct immunofluorescence
2. Indirect immunofluorescence
3. Complement technique

Direct Immunofluorescence

This is the earliest form of the immunofluorescence technique. In this technique, antibodies will directly react with the antigen in the tissue.

Sample Collection and Specimen Transport

For vesiculobullous lesions, the recommended biopsy site is the perilesional area, and a tissue sample of about 3–4 mm is typically sufficient for analysis [8]. The biopsy site should be approximately 3 mm from the blister but not more than 10 mm away [9]. For conditions like oral lichen planus (OLP) and discoid lupus erythematosus (DLE), the lesion itself is biopsied [10]. Once the biopsy is complete, the tissue must be rinsed in distilled water or normal saline to remove blood and proteins and maintain a neutral pH. The tissue should then be transported in Michel's medium, which preserves the sample for up to one month when refrigerated at 4°C. Michel's medium has a shelf life of over six months [11]. If Michel's medium is not available, normal saline can also be used for sample transport, but analysis must be done within 24 hours [10]. Once received in Michel's medium, the sample is washed in phosphate-buffered saline and then processed by freezing, embedding in optimal cutting temperature (OCT) compound, and cutting 4–5-micron sections using a cryostat [12]. While frozen sections are ideal for DIF, formalin-fixed paraffin-embedded sections can also be diagnostically useful [13].

Staining procedure for DIF

1. Wash the sections in 0.1 M phosphate buffered saline (PBS) for 3 changes over 30 minutes [1]. This is to remove unbound protein, so as to reduce the background staining.
2. Drain the excess and bloat it with tissue paper.
3. Add optimally diluted FITC conjugated immunoglobulins (IgG, IgA, IgM, C3b, fibrin) and incubate at 37°C for 45 minutes to 1 hour.
4. Wash the sections with PBS for 3 changes.
5. Mount with glycerine.
6. Keep at 4°C, Stained slides should be kept away from light [14]

Indirect Immunofluorescence

Indirect immunofluorescence is a two-step procedure in which an appropriate substrate tissue sample is first treated with an unlabelled 1° antibody, and then a secondary antibody conjugated with fluorescent dye is used to bind with the primary antibody [15]. The substrate, which contains the corresponding antigen is selected which is commercially available or prepared in the own laboratory. A large number of tissue substrates including monkey esophagus, guinea-pig lip/esophagus, rabbit lip/esophagus, rodent urinary bladder and normal human skin are used [16].

Serum Sample Collection

5–10 ml of the patient's blood is collected in a tube without anticoagulant and serum is extracted after centrifugation. Serial dilutions are made (1:10, 1:20, 1:40).

Staining Procedure for IIF

1. Wash the slides with sections in PBS for 5 minutes.
2. Drain the excess PBS and wipe with a pad of tissue paper.
3. Flood sections with the patient's serum and incubate for 30 minutes.
4. Wash the slides with PBS, 3 washes each for 10 minutes, drain the excess, and wipe with tissue paper.
5. Flood the sections with second stage conjugated antibody and incubate for 30 minutes.
6. Wash the slides in PBS for 10 minutes (3 washes) and drain the excess.
7. Mount the slides in buffered glycerine.
8. Keep the slides at 4°C until reviewing [17].

Complement Technique

This is a modification of indirect immunofluorescence. The complement system is a well-regulated group of proteins that are crucial for defending the host and mediating inflammatory responses [18]. In this technique patient's serum is separated from the collected blood sample, smeared on a substrate, and a specific complement is added. The synthesised fluorescein anticomplement antibodies are used to detect the presence of complement in the tissue [19].

Variants of Immunofluorescence

Salt Split Techniques

Routine DIF procedures cannot always be used to distinguish certain diseases. Both Bullous pemphigoid (BP; with autoantibodies against BP180 and BP230 antigens) and Epidermolysis Bullosa Acquisita (with autoantibodies against collagen VII) produce a linear band of IgG and complement at the dermal–epidermal junction. So, splitting the skin before the immunofluorescent procedure helps these target antigens to be more precisely localized. In this procedure, the skin is incubated in 1 M sodium chloride, which splits the skin at the level of the lamina lucida. Collagen VII immune complexes are retained on the dermal side and BP180 immune complexes on the epidermal side of the sample, enabling the differentiation between these two diseases [20].

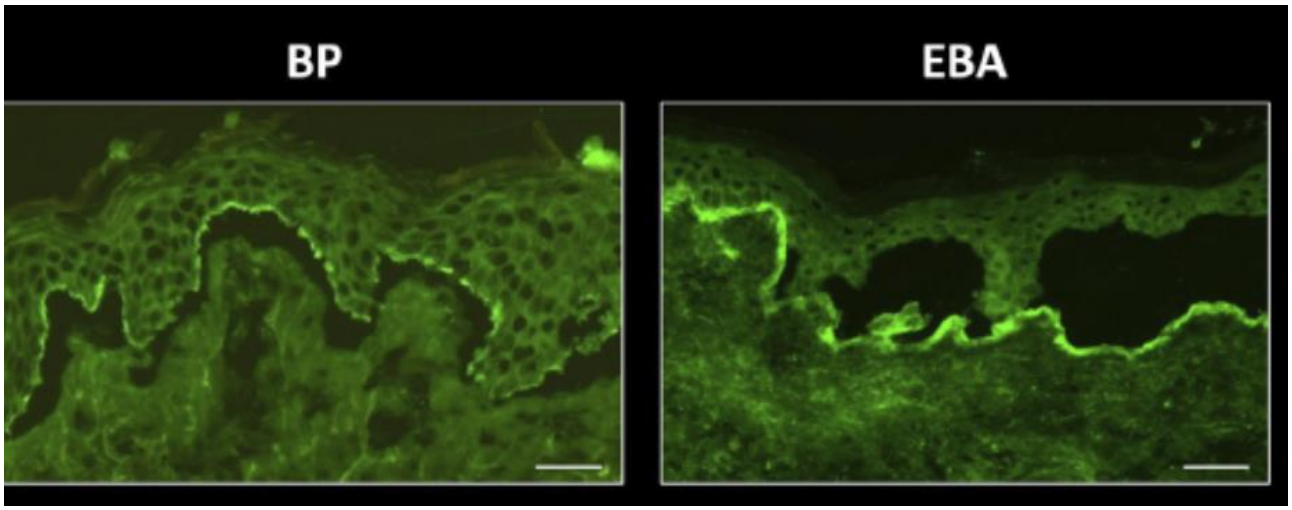


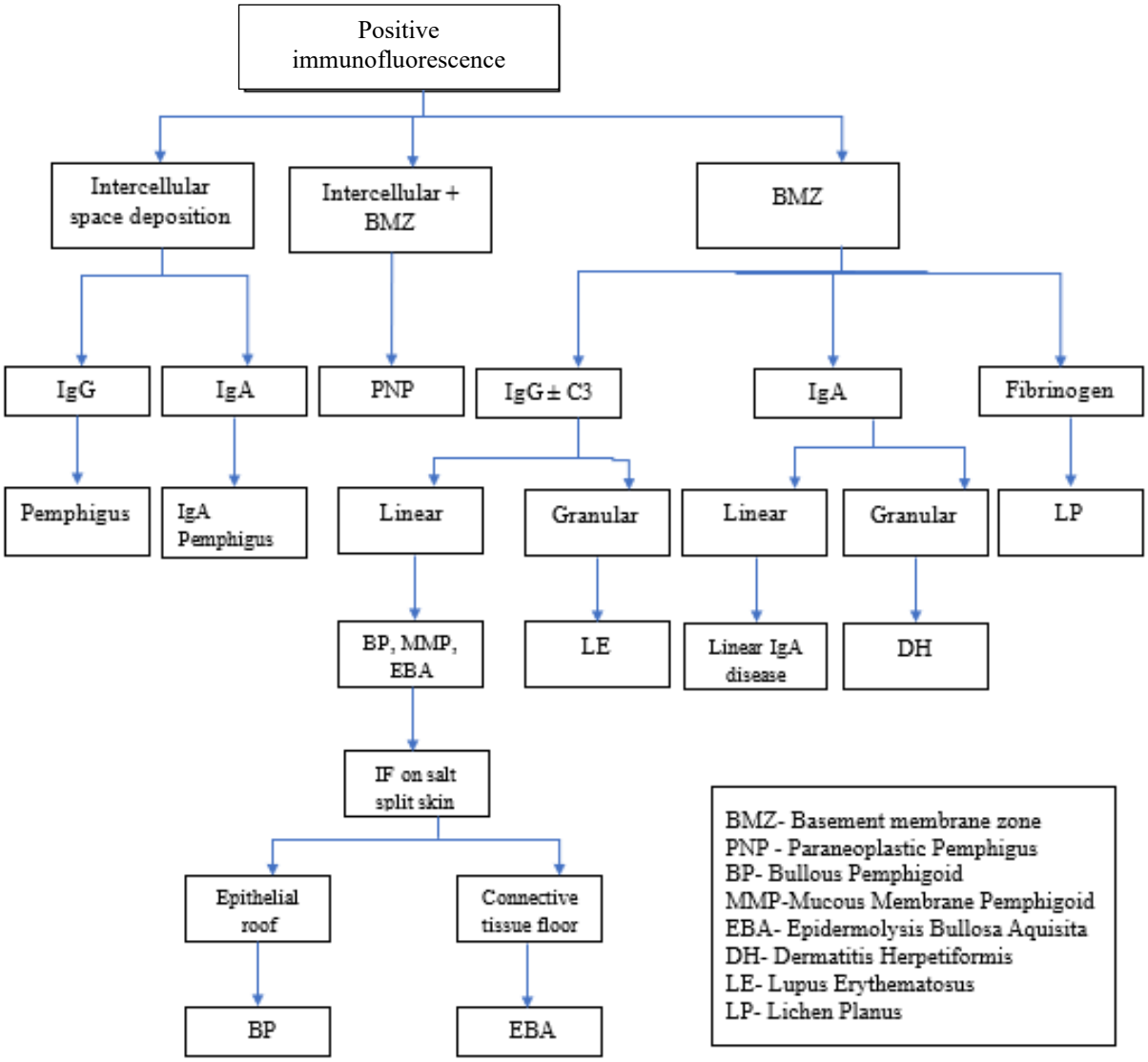
Diagram showing Salt split technique

Pic courtesy: Mee, John. Diagnostic Techniques in Autoimmune Blistering Diseases. British Journal of Biomedical Science.2023 November 80.11809

Interpretation of Immunofluorescent Staining

For interpreting immunofluorescent specimens, important points to consider are:

1. Main site of deposition
2. The class of immunoglobulin deposited
3. The number of immunoreactants.
4. Deposition in other sites besides the main site.



Applications in Oral Lesions

Most of the vesiculobullous lesions present with similar clinical features, like blisters, erosion, ulcers, etc. Therefore, immunofluorescent testing can be used as an adjuvant in establishing the correct diagnosis of bullous diseases, particularly when the clinical and microscopic findings are inconclusive. Vesiculobullous disorders with oral manifestations can be divided into intraepithelial and subepithelial blistering diseases on the basis of the level at which split occurs.

Intraepithelial disorders	Subepithelial disorders
Pemphigus vulgaris (PV) Paraneoplastic Pemphigus (PNP)	Bullous Pemphigoid (BP) Mucous Membrane Pemphigoid (MMP) Epidermolysis Bullosa Aquisita (EBA) Linear IgA disease Dermatitis Herpetiformis (DH) Lupus Erythematosus (LE) Lichen Planus (LP)

Pemphigus

Pemphigus includes a group of autoimmune blistering diseases of the skin and mucous membranes characterized by circulating immunoglobulin G (IgG) antibody directed against the cell surface of keratinocytes. Desmosomal proteins like Desmoglein 3 and 1 are commonly affected. Desmoglein 3 is expressed in the parabasal region of the epidermis and oral epithelium, whereas Desmoglein 1 exhibits little expression in the oral epithelium and is predominantly located in the superficial layer of the epidermis [21]. The primary subsets of Pemphigus include Pemphigus Vulgaris (PV), Pemphigus Foliaceus (PF) Paraneoplastic Pemphigus, Pemphigus Vegetans, and Pemphigus

Erythematosus. Immunofluorescence shows a fishnet or chicken wire pattern of binding of IgG localised to the intercellular spaces [15,19]. The fluorescence patterns seen in PV and PF, as well as their variants, Pemphigus Vegetans and Pemphigus Erythematosus (PE), are similar. IgA Pemphigus shows IgA deposition at intercellular space (ICS). Variation in the intensity of fluorescence at the various layers of the epidermis may be caused by differences in the relative amounts of the target desmosomal proteins for each of the two diseases, namely desmoglein 1 for PF and desmoglein 3 for PV [22].

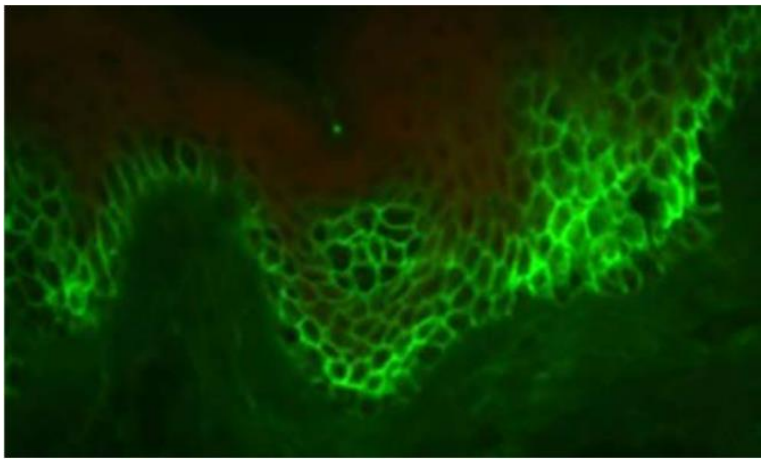


Figure 1: DIF of pemphigus showing intercellular deposition of IgG in fish net pattern.
Courtesy: Porro AM, Seque CA, Ferreira MCC, Enokihara MMSES. Pemphigus vulgaris. An Bras Dermatol. 2019 Jul 29;94(3):264-278.

Paraneoplastic Pemphigus

Paraneoplastic Pemphigus is a very rare variant associated with malignant neoplasm, which was first described by Anhalt *et al.,* in 1990. The most commonly reported malignancies associated with Paraneoplastic Pemphigus are Lymphomatoid and Haematologic, example: B-cell Lymphoma, Chronic Lymphocytic Leukemia, Castleman's disease, and Thymoma [23,24]. Precise etiopathogenesis of these lesion remains unknown, but it could be due to cross reaction of antibodies produced against tumour antigen to host cell. Host lymphocyte produce IL-6 (Interleukin) in response to tumour, this results in abnormal production of antibodies against desmosome and hemidesmosome complex. It can affect both skin and oral mucosa, oral mucosal involvement is early. Clinical presentations include palmar and plantar ulcers, multiple ulcers of oral mucosa, erythema and conjunctival involvement [1]. Histopathology shows acantholysis, band-like infiltrate of lymphocytes subepithelially. In direct immunofluorescence examination, deposition of IgG and C3 in intercellular space and or a linear deposition in basement membrane zone is seen [25] (Figure 2).

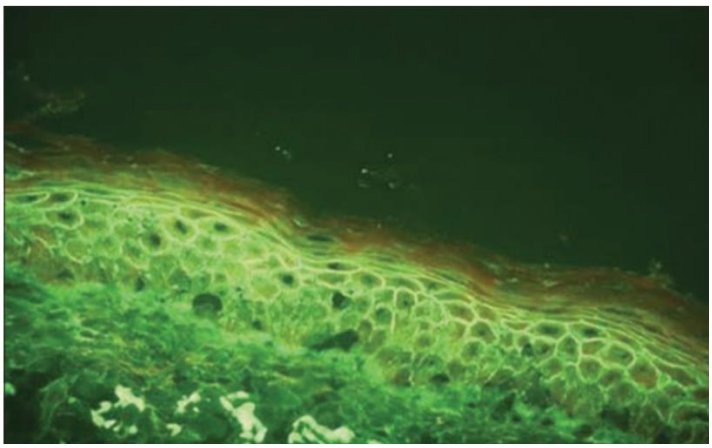


Figure2: Intercellular deposition of IgG in paraneoplastic pemphigus.
courtesy: Nithya SJ, Sankarnarayanan R, Hemalatha VT, Sarumathi T. Immunofluorescence in oral lesions. Journal of Oral Maxillofacial Pathology. 2017 Sep-Dec;21(3):402-406

Mucous Membrane Pemphigoid

Mucous Membrane Pemphigoid is an autoimmune blistering disease that affects the mucous membrane, including the conjunctiva, oral cavity, oropharynx, nares, genitals and skin. Autoantibodies IgG or IgA or both attack molecules in the hemidesmosome.

The major antigens involved in this condition are BP 180 and laminin. The lesions appear as either vesicle or bullae and gingival involvement may present like desquamative gingivitis. Ocular involvement may result in scarring and may eventually result in blindness. Microscopically, it shows subepithelial splits and mild chronic inflammatory infiltrate in the connective tissue. Direct immunofluorescence shows a linear deposit of IgG, IgA, C3 or a combination of these in the basement membrane zone (BMZ) [25, 26] (Figure3).

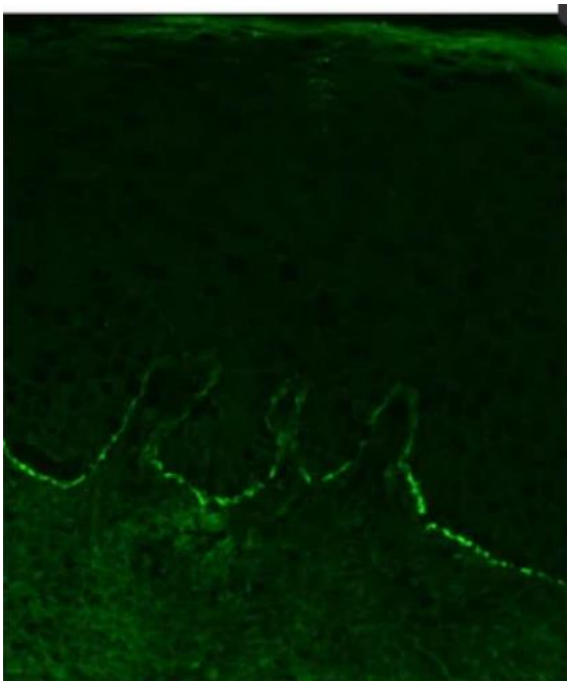


Figure 3: MMP- DIF showing linear deposition of C3 along BMZ.
Courtesy: McFadden JR, Chaudhari AS, Birenbaum D, Margesson L, Gonzalez J, Sriharan A. An Underrecognized Histologic Clue to the Diagnosis of Mucous Membrane Pemphigoid: A Case Report and Review of Diagnostic Guidelines. Dermatopathology (Basel). 2023 Feb 2;10(1):63-69.

Bullous Pemphigoid

Bullous Pemphigoid is the most common autoimmune blistering condition, which is characterised by bullous formation due to autoantibodies directed against the BMZ antigens BP180 and BP 230. The greater incidence is over 60 years of age and usually shows periods of remission and relapse. Pruritus is the early symptom, followed by the formation of tense bullae. Rupture of the bullae results in a crust that eventually heals without scarring. Histopathology shows a subepithelial split with mixed inflammatory infiltrate, eosinophils within the bullae is characteristic. DIF shows linear IgG and or C3 deposits at the basement membrane zone [1, 27] (Figure 4).

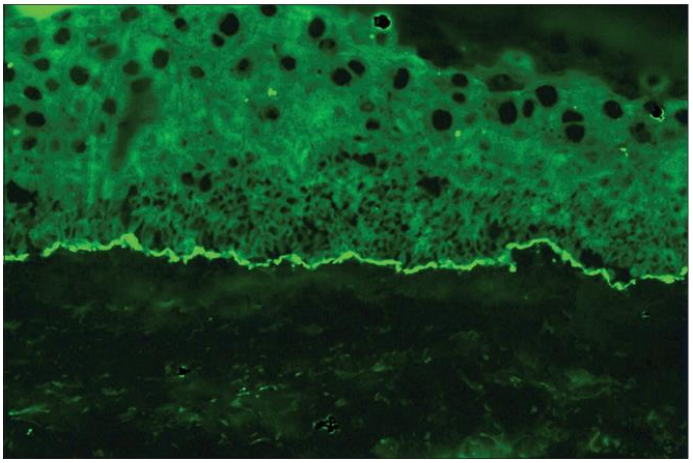


Figure 4: Bullous pemphigoid – DIF showing linear deposits of C3 at BMZ
Courtesy: Mysorekar VV, Sumathy TK, Shyam Prasad AL. Role of direct immunofluorescence in dermatological disorders. Indian Dermatol Online J. 2015 May-Jun;6(3):172-80.

Epidermolysis Bullosa Aquisita (EBA)

It is a rare subepidermal bullous disease with IgG autoantibodies directed against type VII collagen of anchoring fibrils. Anchoring fibrils helps in binding epithelium to underlying connective tissue. Immunological attack of anchoring fibrils results in the formation of subepithelial bullae. Patients present with erosions, blisters, and scars over trauma-prone surfaces such as the dorsal hands, elbows, knees, sacral area, and toes. Affects middle aged or older adults, oral involvement is seen in 50% of cases. Direct immunofluorescence shows linear deposition of IgG along the BMZ. Occasionally, C3, IgA and IgM are also noted [1, 28] (Figure 5).

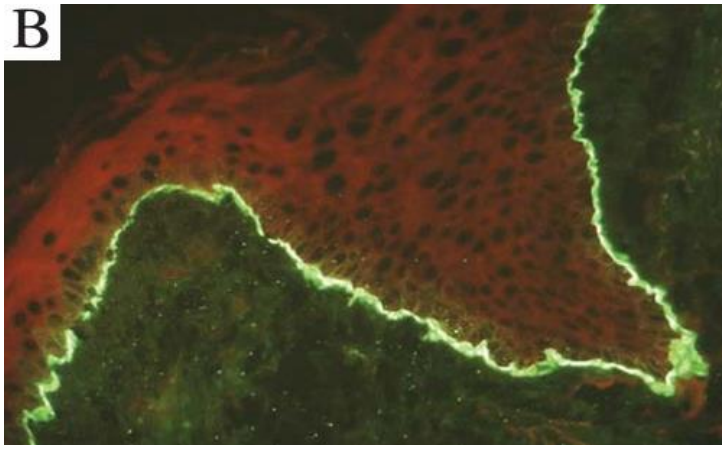


Figure 5: EBA- DIF showing linear IgG deposits in the BMZ
Courtesy: Aoki V, Sousa JX Jr, Fukumori LM, Périgo AM, Freitas EL, Oliveira ZN. Direct and indirect immunofluorescence. An Bras Dermatol. 2010 Jul-Aug;85(4):490-500

Linear IgA Disease

Linear IgA disease is rare subepidermal blistering disease that may be drug induced or idiopathic. It is characterised by IgA antibodies are targeted against the BMZ. It shows bimodal age distribution, in children between 6 months and 10 years of age and adult after 60 years of age. The lesion appears as hemorrhagic or clear bulla with erythematous base. DIF shows linear deposition of IgA along the basement membrane [29,30] (Figure 6).

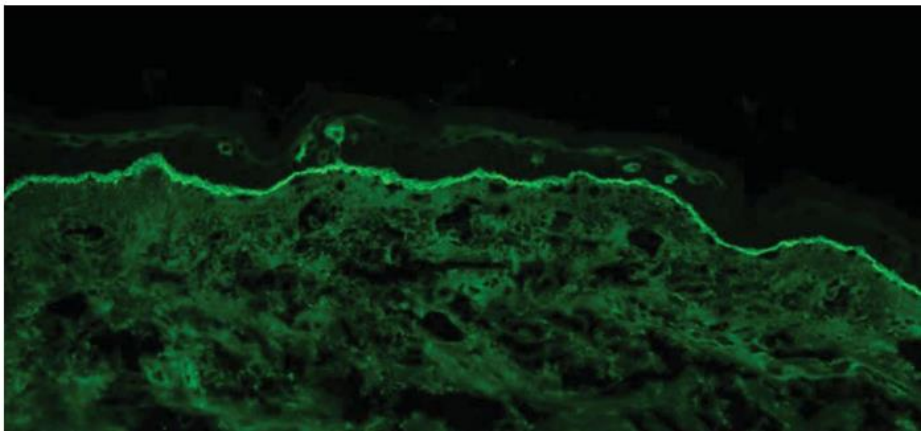


Figure 6: Linear IgA disease: linear IgA deposition along the BMZ
Courtesy: Kim RH, Brinster NK. Practical Direct Immunofluorescence. Am J Dermatopathol. 2020 Feb;42(2):75-85

Dermatitis Herpetiformis

Dermatitis Herpetiformis is chronic autoimmune disease that result in an extremely itchy rash commonly affecting extensor surface. This disease seems to be associated with gluten-sensitive enteropathy. This result from IgA mediated autoimmune response to transglutaminase. Lesions presents as erythematous papules or vesicles usually symmetrical distribution is seen. Microscopic examination shows subepidermal splits with neutrophilic infiltration. DIF shows granular deposition of IgA at BMZ [31] (Figure 7).

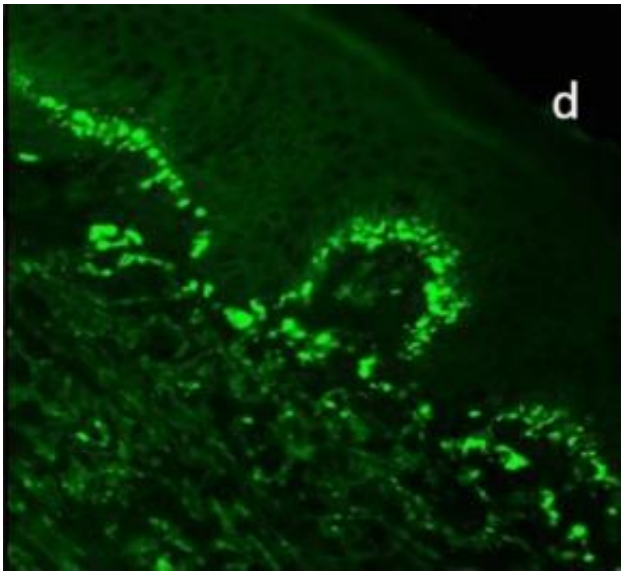


Figure 7: Dermatitis herpetiformis-granular deposition of IgA
Courtesy: Reunala T, Hervonen K, Salmi T. Dermatitis Herpetiformis: An Update on Diagnosis and Management. Am J Clin Dermatol. 2021 May;22(3):329-338

Lupus Erythematosus

Lupus Erythematosus is the common immune mediated collagen vascular disease. Systemic Lupus Erythematosus is a multisystem disease commonly affecting kidney and heart. Oral lesions include ulceration, erythema, hyperkeratosis predominantly affecting palate, buccal mucosa and gingiva along with nonspecific systemic symptoms. 40- 50 % patients show rash having pattern of butterfly on malar area. Histopathologically lesion shows hyperkeratosis, alternating atrophy and thickening of the spinous cell layer, degeneration of the basal cell layer, and subepithelial lymphocytic infiltrate, usually deep perivascular infiltration. DIF shows the deposition of one or more immunoreactant usually IgG, IgM, C3 in a shaggy or granular band at the BMZ [1] (Figure 8).

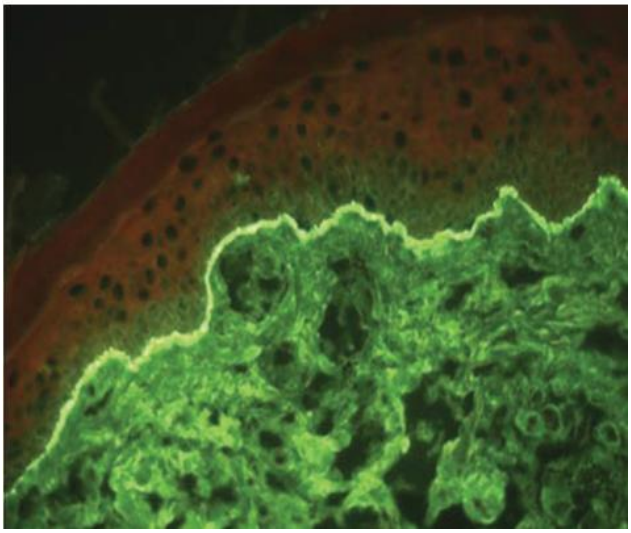


Figure 8: LE- linear deposition of C3 along the BMZ
Courtesy: Aoki V, Sousa JX Jr, Fukumori LM, Périgo AM, Freitas EL, Oliveira ZN. Direct and indirect immunofluorescence. An Bras Dermatol. 2010 Jul-Aug; 85(4):490-500.

Lichen Planus

Lichen Planus (LP) is a chronic idiopathic immune-mediated inflammatory disorder involving skin, hair, nails, and mucosae (oral, genital, esophageal, and ocular). The term Lichen Planus is derived from the Greek word lichen for “tree moss” and the Latin word planus for "planar." LP is a heterogeneous disease with widely varying clinical presentations having different natural history, prognosis, sequelae, and outcomes**.** The Oral Lichen Planus presents as reticular, papular plaque or verrucous form or erosive type. The exact etiopathogenesis is unknown. However, four major areas that play a role in the etiopathogenesis of Lichen Planus are immune dysregulation, infections, environmental

factors, and genetic factors. It is a T cell mediated autoimmune disease in which autoantibodies are targeted against basal cell keratinocytes. The microscopic examination shows saw tooth shape rete ridges, degeneration of the basal epithelial layer, and band like infiltrate predominantly of lymphocytes immediately subjacent to the epithelium. The degenerating keratinocyte may be seen in the area of epithelium and connective tissue interface, termed colloid bodies. DIF shows shaggy fibrinogen at the basement membrane zone with IgM deposition on the colloidal bodies [32, 33] (Figure 9).

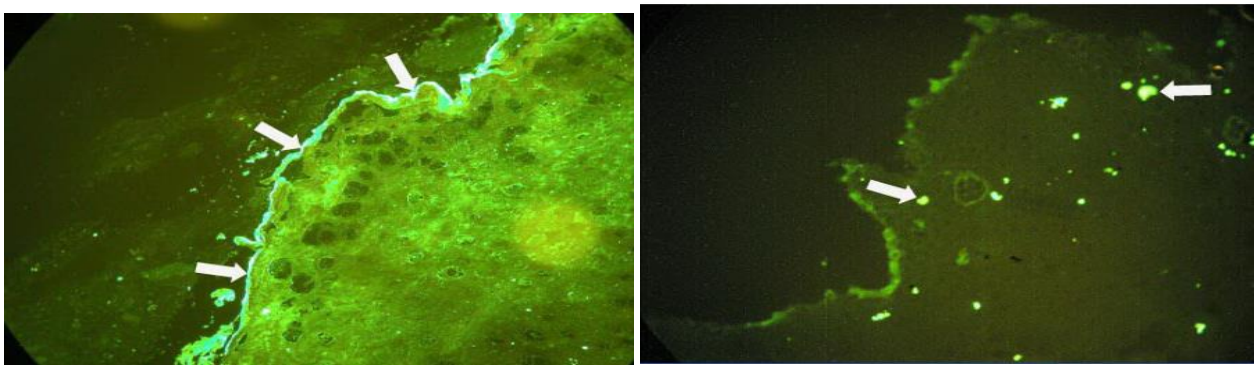


Figure 9: LP-(a)-DIF shows deposition of fibrinogen at BMZ. (b) showing deposition of IgM on colloid bodies in connective tissue

Courtesy: Buajeeb W, Okuma N, Thanakun S, Laothumthut T. Direct Immunofluorescence in Oral Lichen Planus. J Clin Diagn Res. 2015 Aug; 9(8): ZC347

CONCLUSION

Immunofluorescence is a crucial technique in the diagnosis of various immunobullous lesions, as most of the oral lesions may present with similar features. It is a method that helps in the visualisation of antigens in cells through the use of fluorescent dyes. It is considered as the gold standard for the diagnosis of immunobullous disorders and also it helps in monitoring the prognosis of the disease.

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