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Review Article

Immunofluorescence as an investigative tool in diagnosis of Oral mucosal lesions- A Review

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ABSTRACT:

Oral mucosal vesiculobullous disorders are autoimmune blistering disorders in which autoantibodies are directed against antigens present in the epidermis and dermoepidermis junction. These lesions resemble each other clinically and routine biopsies may offer histological similarities. Nowadays immunofluorescence is being used with routine histology to accurately diagnose such lesions. In this article, we present application of immunofluorescence in the diagnosis of oral mucosal lesions namely pemphigus, pemphigoid, oral lichen planus, lupus erythematosus, epidermolysis bullous acquisita and linear IgA disease. A brief outline of each disease, in terms of its underlying pathophysiology, some clinical features is also provided so that the relevance of the immunofluorescence finding may be better understood. The 2 main methods of immunofluorescence labelling are direct and indirect along with 2 newer techniques the salt split and biochip immunofluorescence testing can add to the certainty of diagnosis.

Key words: Immunofluorescence, Direct immunofluorescence, Indirect immunofluorescence, Oral mucosal lesions

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INTRODUCTION

The practice of pathology is currently undergoing significant change, in large part due to advances in analysis of DNA, RNA, and proteins in tissues. These advances have permitted improved biologic insights into many developmental, inflammatory, metabolic, infectious, and neoplastic diseases. Moreover, molecular analysis has also led to improvements in the accuracy of disease diagnosis and classification. It is likely that, in future, these methods will increasingly enter into the day-to-day diagnosis and management of patients.[1] There are various diagnostic tools which are used for the diagnosis of many diseases such as direct immunofluorescence (DIF), indirect immunofluorescence (IIF), enzyme linked immunosorbent assay (ELISA), immunoblotting, biochip immunofluorescence test and salt split immunofluorescence. The present review highlights the principle of Immunofluorescence and its importance in diagnosing various mucosal lesions.

Immunofluorescence (IF) is a reliable biochemical staining technique for the detection of antibodies, which are bound to antigen in the tissue; or circulating in body fluids. [2] IF is a fluorescent staining method which uses antibodies conjugated to a fluorescent labeller (fluorochromes, enzymes, radioactive compounds) to visualize specific proteins/antigens in cell or tissue sections. [3]

The relative simplicity and accuracy of the technique has made IF a powerful technique in the diagnosis of autoimmune diseases. [4] The principle of IF takes advantage of the fundamental structure of all atoms, where electrons are arranged in discrete energy levels around the atomic nucleus.[5] Fluorescence is the property of absorbing light rays of one particular wavelength and emitting rays with a different wavelength.[6] The two main methods of

immunofluorescent labelling are direct and indirect. Less frequently used is direct IF whereby the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect IF, the unlabelled antibody specific for the molecule of interest is called the primary antibody and a second anti-immunoglobulin antibody tagged with fluorescent dye is directed towards the constant portion of the first antibody is called the secondary antibody.[7,8]

IF studies are considered the 'gold standard' for diagnosis of autoimmune diseases. This technique was first described by Albert Coons and colleagues back in 1941, where they successfully produced fluorescein-conjugated antipneumococcal-3 antibodies to detect type 3 Streptococcus pneumonia.[9,10] A discovery which made possible to observe microscopically antigens, antibodies and their related substances on tissue sections or on cell smears.[11] The substance initially used by Coons was beta-anthracene, which produces blue fluorescence. [2] Fluorochromes, currently used are fluorescein isothiocyanate (FITC) which produces apple-green color; tetramethylrhodamine isothiocyanate (TRITC) with a red colour of fluorescence; and phycoerythrin, which also shows red fluorescence. [2] These markers are detected with a fluorescence microscope equipped with a mercury vapor or xenon light source, and appropriate exciter and barrier filters. The exciter filter serves to shed light of necessary wavelength on the examined slide, while the barrier filter stops the exciting photons, letting through only the fluorescent light. [2] In the past, every laboratory had to produce its own fluorochrome-labeled antibodies. Nowadays, a wide range of ready-to-use conjugates, suitable for clinical and research work, are available commercially [2].

Initially, most of the efforts were made in the purification of antisera, search of ideal labelling markers, improvement in cryostat sectioning, better fluorescent microscopy and increasing the sensitivity of microphotography.[11] Beutner and Jordon in 1964, made use of IF technique by demonstrating antibodies in the sera of pemphigus patients, by IIF. [12] In 1971, Jordon et al. performed DIF on lesional and perilesional skin of patients suffering from oral mucosal lesions to demonstrate the deposition of IgG antibodies at the inter-cellular spaces in the epidermis. [12] During the ensuing years, newer substrates and modified substrate e.g. salt-split specimens used for DIF and IIF to enhance the sensitivity and specificity of the technique have come into being. [13]

Principle of Fluorescence

Fluorescence and phosphorescence are two types of luminescence. When molecules with luminescent properties absorb light, they emit light of a different wavelength. With fluorescence the emission of light occurs extremely rapidly after the absorption of excitation light, whereas with phosphorescence emission continues for milliseconds to minutes after the energy source has been removed.[14] Fluorescence is the property of absorbing light rays of one particular wavelength and emitting rays with a different wavelength. Fluorescent dyes show up brightly under ultraviolet light as they convert ultraviolet into visible light.[6] Fluorescent materials give off light because of their atomic structure. Electrons are arranged in discrete energy levels surrounding the atom's nucleus with each level having a predetermined amount of energy. When an electron absorbs the energy from a photon of light it becomes "excited" and jumps to a higher, less stable energy level. The excited state does not last long. The half-life of the excited state is generally less than 10 seconds. The electron loses a small amount of energy as heat and the remainder of the extra energy is given off in the form of a photon. The emitted fluorescence has a lower energy than the absorbed light, so the wavelength of the emitted light is longer than that of the excitation light. [15,16]

The ideal fluorochrome would be a molecule with the following properties: (a) An absorption peak at an excitation wavelength available on the fluorescence detection instrument. (b) Bright fluorescence with high quantum yield. (c) A narrow emission spectrum that falls within one of the instrument's detection bands. (d) Good photostability. (e) Fluorescence properties that are not significantly altered by conjugation to an antibody or by the local environment of the sample.[16]

APPEARANCES OF VARIOUS ORAL MUCOSAL LESIONS

Pemphigus

Pemphigus is a group of blistering autoimmune diseases that affects the skin and mucous membranes of the oral cavity. Worldwide, this condition affects fewer than 5 in 1,000,000 people every year but if left untreated, pemphigus can be life-threatening due to the increased risk of skin infection, sepsis, and dehydration.[17] Pemphigus occurs due to the presence and circulation of autoantibodies against desmoglein (Dsg), a cadherin-type cell adhesion molecule that forms desmosomes and binds keratinocytes together. Thus, keratinocytes separate from each other (acantholysis) in the suprabasal layer of the stratified squamous epithelium and this presents clinically as superficial skin blisters which easily rupture and heal poorly. Of all the variants of pemphigus, Pemphigus Vulgaris (PV) is the most common type. PV is the type that usually affects the mouth because the oral mucosa mainly expresses Dsg3, whereas Dsg1 is poorly expressed. [18] The pattern of fluorescence in PV is the deposition of IgG around epidermal cells. [16] Williams in 1989 stated that DI performed on perilesional tissue reveals a uniform fishnet pattern of binding of IgG localized to the intercellular spaces. [19] Parlowsky et al. in 2003

stated that DIF reveals the deposition of complement (C3) and IgG, IgA, or IgM, within the intercellular spaces of epithelium resulting in a reticular pattern diagnostic of pemphigus. [20] IIF performed on a monkey esophagus demonstrated the presence of circulating IgG auto antibodies that bound to the epithelium with an intercellular staining pattern. [21] Mutasim et al. in 2001 stated that a punctate or granular fluorescence is appreciated at higher magnification. The pattern of fluorescence is same for all types of pemphigus. [22] Challacombe et al. in 2001 stated that assay of serum antibody titers by IIF may also help to guide in prognostication and therapy. [23]

Mucous Membrane Pemphigoid

Pemphigoid is a group of autoimmune skin diseases characterised by subepithelial blistering. Types of pemphigoid include bullous pemphigoid, mucous membrane pemphigoid (previously known as cicatricial pemphigoid), and pemphigoid gestationis. Of these, mucous membrane pemphigoid (MMP) is the main type that affects the oral mucosa. [24,25] MMP occurs due to the presence and circulation of autoantibodies against hemidesmosomal components in the basement membrane zone (BMZ), such as the β4 subunit of α6β4 integrin, laminin-5, laminin-6, type VII collagen, and bullous pemphigoid antigens 1 and 2 (BP230 and BP180 respectively). [26,27]

Deposition of IgG, C3, or both at the basement membrane zone is seen in Bullous pemphigoid. Deposition of C3 with significantly higher intensity than IgG strongly favors the pemphigoid group of diseases. [14] Jordan et al. in 2002 stated that deposition of C3 in the BMZ is detected in almost all patients. [28] DIF of perilesional tissue in MMP-positive cases typically reveals a linear, homogenous, ribbon-like deposition of IgG and/or C3 (and occasionally IgA) along the BMZ. Ahmed & Hombal (1986) collected data from nine studies and reported that DIF detected immunoglobulin deposition at the BMZ in 84% (90/107 cases) of oral mucosal specimens. [29] Circulating antiBMZ antibodies (IgG isotype) can also be occasionally detected by IIF, usually only when mucosal substrates are used. [30] This corresponds with the clinical presentation of subepithelial blistering in MMP, where it mainly affects mucous membranes and rarely the skin. However, these circulating autoantibodies are generally difficult to detect, as is evident from the study by Ahmed & Hombal (1986) which found positive IIF findings in only 19% (28/144) of cases. [29] Suggested reasons for this are that (a) MMP is a localized disease, hence only small quantities of anti-BMZ antibodies are produced with most of them being bound, and (b) routine indirect IF techniques are not sensitive enough to detect very low titres. [31-33]

Linear IgA Disease

Linear IgA disease (LAD) is an autoimmune skin disease characterized by subepithelial blistering of skin and mucous membranes which are similar in appearance to other blistering diseases, such as pemphigoid and dermatitis herpetiformis. LAD is extremely rare with an estimated incidence of 5 in 10,000,000 people in Western Europe, affecting both children and adults. [34] It usually initiates spontaneously but can be triggered by certain drugs or medications, such as vancomycin. [35-38] LAD occurs due to the presence and circulation of IgA autoantibodies against antigens in the BMZ, such as BP180, BP230, and LAD285 [39] The characteristic feature of LAD-positive samples by DIF analysis is the linear deposition of IgA along the BMZ. [40] However, there may be additional involvement of other immunoreactants, such as C3, IgG, and very rarely IgM, in a small number of cases. [34,40,41] Circulating anti-BMZ IgA antibodies may occasionally be detected by IIF in low titres, ranging from 1:2 to 1:64. [41] The morphology of IgA deposition in Linear IgA disease is similar to deposition of other immunoreactants along the BMZ in other subepidermal bullous diseases such as bullous pemphigoid and epidermolysis bullosa acquisita. [14] But exclusive deposition of IgA alone is extremely helpful in the diagnosis of Linear IgA disease. [16]

Oral lichen planus

Oral lichen planus (OLP) is a chronic inflammatory autoimmune disease that affects the mucous membranes of the oral cavity, presenting clinically as white lacy lesions, papules, or plaques; sometimes resembling keratotic diseases (e.g. leukoplakia). [42] The causes or triggering factors of OLP are largely unclear, but one key early event in the pathophysiology of OLP is the increased production of Th1 cytokines, which leads to the activation and migration of T cells to the oral epithelium. [42,43] There, the T cells bind to keratinocytes and IFN-γ, followed by the upregulation of metalloproteinase-1 (MMP1), MMP3, and p53. [44-46] Thus, apoptosis is induced and destruction of epithelial basal cells ensues.

In OLP-positive cases, DIF typically reveals linear fibrinogen deposition along the BMZ, extending into the papillary lamina propria in a 'shaggy' pattern. [47] Additionally, deposition of IgM, IgA, IgG, and C3 on cytoid bodies at the BMZ or papillary lamina propria may be detected. [47,48] The presence of both these features are usually required for diagnosis of OLP by DIF. [48,49] Ig deposits along the BMZ are rarely seen in OLP, occurring only in 3-30% of cases. [50]

Regezi and Scuibba in 1998 stated that DIF study demonstrated the presence of fibrinogen along the basement membrane zone in 90%– 100% of cases. [51]

Lupus Erythematosus

Lupus erythematosus (LE) is a term given to a group of chronic autoimmune diseases that can affect many different organs in the body. The exact pathogenesis of LE remains unclear but the potential complex mechanisms involved are described in the literature. [52,53] Oral mucosal manifestations can occur either with systemic LE (SLE), discoid LE (DLE), or alone. These oral lesions have a wide range of clinical presentations, from white keratotic striae resembling OLP to erythematous patches and ulcerations. [54] DIF may be helpful in distinguishing among the various subsets of LE since the frequency of deposition, its morphology, and site of deposition vary among the various subsets of LE. Immune deposits in discoid lupus erythematosus are characteristically found along the dermoepidermal junction. The immunoglobulins most frequently present in cytoid bodies are IgM and IgA. Complement and IgG are less frequently seen. Several patterns of fluorescence along the dermoepidermal junction have been described and include linear, granular, and shaggy pattern. [55,56] The immune deposits most frequently found along the dermoepidermal junction are IgG, IgM, IgA, and C3 in SLE. These immune deposits are characteristically found in combination. [16] Patients with DLE more commonly exhibit fibrinogen deposition (89% of cases) compared to patients with SLE (67%), but cytoid bodies are more common in SLE cases (43%) than in DLE (27%). [50]

Epidermolysis Bullosa Acquisita

Epidermolysis bullosa acquisita (EBA) is a rare, acquired autoimmune disease characterized by subepithelial blistering. The clinical presentation of EBA is similar to that of the dystrophic forms of hereditary epidermolysis bullosa (EB), which is why EBA was historically considered part of the EB group of diseases. [57] EBA occurs due to the presence and circulation of autoantibodies against collagen VII (C7), a major component of anchoring fibrils (AF) that functions to link the BMZ lamina densa to the papillary lamina propria. Multiple deposits at the BMZ is pattern of deposition strongly favors Epidermolysis bullosa acquisita. In EBA, intense IgG deposition is almost consistently present. The intensity of C3 deposition is usually less than that of IgG. Deposition of IgA is present in approximately two thirds of cases and deposition of IgM in approximately one half of cases. [16]

CONCLUSION

Conventional histopathology and immunological test like direct and indirect immunofluorescence are important technique for the investigation of patients with vesiculobullous diseases. Immunofluorescence plays an important role in diagnosis as well as understanding the pathophysiology. When IF was first described by Albert Coons and colleagues in 1941, it was used to detect one species of bacteria – type 3 Streptococcus pneumoniae – as a proof of concept. Today, 78 years later, it's being used into an immensely wide range of fields, including oral medicine. Immunofluorescence testing is invaluable in confirming a diagnosis that is suspected by clinical or histologic examination, and has enabled treatment and management to be more targeted and efficient, resulting in improved patient outcomes. IF will be a major tool for diagnosis for many years to come that any pathologist studying cells & molecules cannot afford to ignore.

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