Oral Surgery, Oral Medicine, Oral Radiology, 2014, Vol. 2, No. 1, 6-10
Available online at http://pubs.sciepub.com/oral/2/1/2

DOI:10.12691/oral-2-1-2

Immunofluorescence in Oral Mucosal Diseases –A Review

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Received January 16, 2014; Revised February 12, 2014; Accepted February 20, 2014

Abstract Oral mucosal vesiculobullous and ulcerative lesions are frequently present diagnostic problems because the lesions may resemble each other clinically and routine biopsies may offer histological similarities and diagnosis of nonspecific inflammation. Thus, immunofluorescence is increasingly being used with routine histology to accurately diagnose these lesions. Immunofluorescence is a reliable biochemical staining technique for the detection of antibodies, which are bound to antigen in the tissue or in circulating body fluids. The relative simplicity and accuracy of the technique has made immunofluorescence a powerful technique in the diagnosis of bullous diseases. The diagnosis of oral mucosal diseases requires clinicopathological correlation and immunofluorescence methods provide a useful adjunct to light microscopy. The two main methods of immunofluorescent labelling are direct and indirect. Immunofluorescence testing can add to the certainty of diagnosis, sometimes modify it and occasionally reveal a differential diagnosis.

Keywords: *direct immunofluorescence, indirect immunofluorescence, autoimmune bullous diseases*

Cite This Article: Priyanka Sawant, Avinash Kshar, Raghvendra Byakodi, and Arati Paranjpe, "Immunofluorescence in Oral Mucosal Diseases –A Review." *Oral Surgery, Oral Medicine, Oral Radiology*, vol. 2, no. 1 (2014): 6-10. doi: 10.12691/oral-2-1-2.

1. Introduction

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. [1] The immunobullous disorders are a group of autoimmune diseases in which components of the epidermis and basement membrane zone are targeted, resulting in the formation of cutaneous and mucosal blisters. The diagnosis of these autoimmune oral mucosal diseases requires clinicopathological correlation and immunofluorescence methods provide a useful adjunct to light microscopy. [2] The relative simplicity and accuracy of the technique has made immunofluorescence a powerful technique in the diagnosis of autoimmune diseases. [3] 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. [4] The two main methods of immunofluorescent labelling are direct and indirect. Less frequently used is direct immunofluorescence whereby the antibody against the molecule of interest is chemically conjugated to a fluorescent dye. In indirect immunofluorescence, 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. [5,6] (Figure 1).

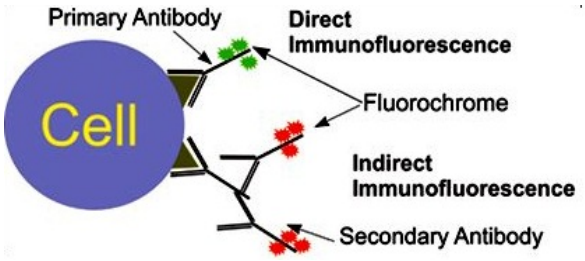

Figure 1. Direct and indirect immunofluorescence showing fluorochrome dye labelled antibodies

2. History

Immunofluorescence studies are considered the 'gold standard' for the diagnosis of autoimmune blistering diseases. However, it was not before 1941 when Coons *et al*. developed the immunofluorescence techniques for the first time. A discovery which made possible to observe microscopically antigens, antibodies and their related substances on tissue sections or on cell smears. [7] Beutner and Jordon in 1964, made use of this newly introduced technology by demonstrating antibodies in the sera of pemphigus patients, by indirect imunofluorescence. [8] Jordon *et al*. performed direct immunofluorescence on lesional and perilesional skin in 1971 to demonstrate the deposition of IgG antibodies at the inter-cellular spaces in the epidermis. [8] Initially, most of the efforts were made in the purification of antisera, search of ideal labeling

markers, improvement in cryostat sectioning, better fluorescent microscopy and increasing the sensitivity of microphotography. [7] During the ensuing years, newer substrates and modified substrate e.g. salt-split specimens used for direct and indirect immunofluorescence to enhance the sensitivity and specificity of the technique [9].

3. Basis of Immunofluorescence

In immunofluorescence technique antigens, antibodies or their complexes are viewed under an ultraviolet microscope, using the corresponding antibodies complexed to a fluorochrome. Fluorochromes are the substances that have electrons which, when irradiated with light of certain wavelength, achieve an unstable higher energetic state. On returning to their basic state, as a spontaneous process, they emit light with a characteristic longer wavelength. The substance initially used by Coons was beta-anthracene, which produces blue fluorescence. [1] 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. [1] 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. [1] 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 [1].

4. 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. 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. [10,11] A range of wavelengths of light can excite the electrons of a fluorochrome. The light produced by fluorochromes has a range of wavelengths. The emission of light from fluorescein ranges from 490 nm to 630 nm, and the emission peak is approximately 515 nm. Since the phenomenon of fluorescence was first explained by a British scientist, Sir George Stokes, in 1852, the shift in wavelength from short to long during fluorescence is called "Stokes shift". [11] 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 [11].

5. Appearances of Various Oral Mucosal Lesions

A. Pemphigus Vulgaris

The intercellular space fluorescence pattern results from binding of antibodies to desmosomal proteins around the keratinocyte cell surface and is characteristic of the pemphigus group of disorders. The pattern of fluorescence in Pemphigus vulgaris is the deposition of IgG around epidermal cells(Figure 2.A). [11] Williams in 1989 stated that direct immunofluorescence performed on perilesional tissue reveals a uniform fishnet pattern of binding of IgG localized to the intercellular spaces. [12] Parlowsky *et al*. in 2003 stated that direct immunofluorescence 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. [13] Indirect immunofluorescence performed on a monkey esophagus demonstrated the presence of circulating IgG auto antibodies that bound to the epithelium with an intercellular staining pattern. [14] 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. [15] Challacombe *et al*. in 2001 stated that assay of serum antibody titers by indirect immunofluorescence may also help to guide in prognostication and therapy [16].

B. Paraneoplastic Pemphigus

Deposition of IgG in the intercellular space and basement membrane zone is seen in Paraneoplastic pemphigus(Figure 2.B). Patients with Paraneoplastic pemphigus have antibodies to basement membrane zone proteins in addition to antibodies to desmosomal proteins. The pattern of fluorescence at the basement membrane zone is similar to that seen in Bullous pemphigoid. In the absence of clinical or Histologic information, it is difficult to distinguish between some cases of pemphigus erythematosus and Paraneoplastic pemphigus. Frequently the intercellular space deposition in Paraneoplastic pemphigus is weak or diffuse and nonspecific [11].

C. Bullous Pemphigoid

Deposition of IgG, C3, or both at the basement membrane zone is seen in Bullous pemphigoid(Figure 2.C). Deposition of C3 with significantly higher intensity than IgG strongly favors the pemphigoid group of diseases.

The pattern of deposition in Bullous pemphigoid has been described as linear, wavy, tubular, and granular. The variation in pattern may result from variations in the angle at which the cryosections are made, the intensity of deposition, and the site of biopsy. [11] Jordan *et al*. in 2002 stated that deposition of C3 in the basement membrane zone is detected in almost all patients [17].

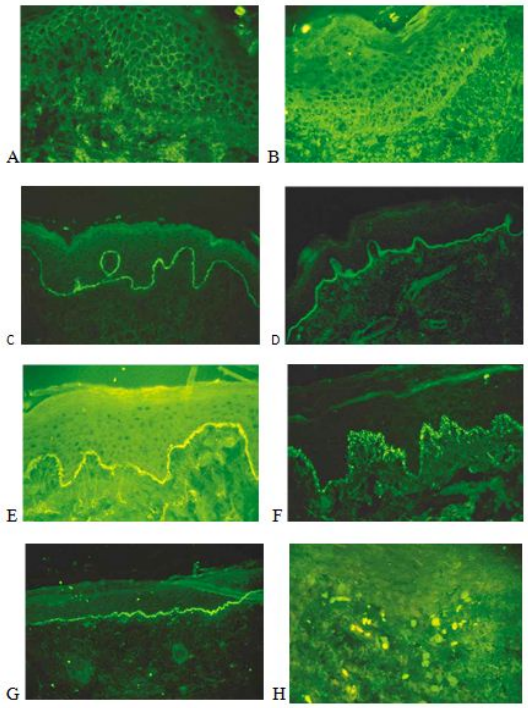

Figure 2. A. Pemphigus vulgaris. DIF: Note deposition of IgG around epidermal cells. **B.** Paraneoplastic pemphigus DIF: Note faint deposition of IgG around epidermal cells. **C.** Bullous pemphigoid. DIF: Note continuous deposition of C3 along the epidermal basement membrane. **D**. Epidermolysis bullosa acquisita. DIF: Note continuous homogeneous deposition of C3 along the BMZ. **E.** Linear IgA disease. DIF: Note deposition of IgA along the BMZ.. **F.** Dermatitis herpetiformis. DIF: Note deposition of IgA in a granular pattern along the BMZ and dermal papillae. **G.** Lupus erythematosus. DIF: Note continuous linear deposition of C3 along the BMZ. **H.** Lichen planus. DIF: Note deposition of IgM within scattered cytoid bodies in the papillary dermis

D. Epidermolysis Bullosa Acquisita

Multiple deposits at the basement membrane zone is pattern of deposition strongly favors Epidermolysis bullosa acquisita (Figure 2.D). In epidermolysis bullosa acquisita, 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 [11].

E. Linear IgA Disease

Linear deposition of IgA at the basement membrane zone is characteristic of Linear IgA disease (Figure 2.E). Deposition of C3 is present less frequently and with lower intensity compared with IgA. The morphology of IgA deposition in Linear IgA disease is similar to deposition of other immunoreactants along the basement membrane zone in other subepidermal bullous diseases such as bullous pemphigoid and epidermolysis bullosa acquisita. But exclusive deposition of IgA alone is extremely helpful in the diagnosis of Linear IgA disease [11].

F. Dermatitis Herpetiformis

Granular deposition of IgA and C3 in the papillary dermis and along the basement membrane zone is diagnostic of Dermatitis herpetiformis (Figure 2.F). Deposition of IgA is present in 100% of patients when the biopsy specimen is obtained from normal appearing perilesional skin. Deposition of C3 is seen in approximately half of cases. Deposition of IgG or IgM, or both, is less frequent and less intense [11].

G. Lupus Erythematosus

Direct immunofluorescence may be helpful in distinguishing among the various subsets of lupus erythematosus since the frequency of deposition, its morphology, and site of deposition vary among the various subsets of lupus erythematosus. The combination of IgG and IgM favors the diagnosis of discoid lupus erythematosus. 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. [18,19] The continuous linear deposition of C3 along the basement membrane zone is seen in Lupus erythematosus (Figure 2.G). The immune deposits most frequently found along the dermoepidermal junction are IgG, IgM, IgA, and C3 in systemic lupus erythematosus. These immune deposits are characteristically found in combination [11].

H. Lichen Planus

In certain cases in which the clinical and histologic findings are not characteristic direct immunofluorescence, may be helpful. Direct immunofluorescence is helpful in differentiating mucosal Lichen planus from other mucosal erosive and bullous diseases such as mucosal pemphigoid. Immune deposits are present within cytoid bodies in the superficial dermis, as well as along the dermoepidermal junction. The most frequently present immune deposits are IgM and fibrinogen. Deposition of IgM within cytoid bodies in the papillary dermis is diagnostic of Lichen planus (Figure 2.H) Some findings that may help favour Lichen planus include the tendency for cytoid bodies in cluster, to be present in high number, to be larger, to have higher fluorescence intensity, and to contain multiple immune deposits [11].

Regezi and Scuibba in 1998 stated that direct immunofluorescence study demonstrated the presence of fibrinogen along the basement membrane zone in 90%–100% of cases [20].

6. Advantages of Direct Immunofluorescence

Immunofluorescence is a relatively simple and reproducible technique. The main advantage of the

technique is the short procedure time. The complete procedure including the preparation of sections, their staining and observation can be performed in one to three hours. Another characteristic is its sensitivity. The sensitivity of the direct method seems to correspond to that of the classical complement fixation test. In cases where one has multiple antibodies raised in the same species, for example two mouse monoclonals, a direct labeling may be necessary [5,9].

7. Disadvantages of Direct Immunofluorescence

Direct immunofluorescence has lower signal and generally higher cost. It has less flexibility. There are difficulties with the labelling procedure in direct immunofluorescence when labelled direct conjugates are unavailable [5,9].

8. Advantages of Indirect Immunofluorescence

Sensitivity of the indirect or complement method is estimated to be between five and ten times higher than that of the direct one. Immunofluorescence besides the diagnostic importance, has a substantial prognostic value, particularly for pemphigus cases.[1] There is amplification of the signal in indirect immunofluorescence because more than one secondary antibody can attach to each primary. Commercially produced secondary antibodies are relatively inexpensive, available in an array of colors, and quality controlled [5,9].

9. Disadvantages of Indirect Immunofluorescence

There is potential for cross reactivity in indirect immunofluorescence. The need to find primary antibodies that are not raised in the same species, while performing multiple labelling study. Samples with endogenous immunoglobulin may exhibit a high background [5,9].

10. Limitations of Immunofluorescence Techniques

10.1. Photobleaching

Photobleaching is the photochemical destruction of a fluorophore due to the generation of reactive oxygen species in the specimen as a byproduct of fluorescence excitation. [5] Photobleaching can be minimized by: (a) decreasing the excitation light in both intensity and duration. (b) reducing the availability of singlet oxygen by the addition of singlet oxygen scavengers. (c) using a low concentration of a fluorochrome with high quantum efficiency [5].

10.2. Autofluorescence

Biological autofluorescence in mammalian cells due to flavin coenzymes and reduced pyridine nucleotides can be problematic in the detection of fluorescence probes in tissues and cells. Fixation with aldehydes, particularly glutaraldehyde, can result in high levels of autofluorescence [5].

10.3. Fluorescence Overlap

One of the problem that must be dealt with when measuring fluorescence of more than one color is the possibility that the emission signals overlap. It is necessary to remove the overlapping signal or it will give a false level for one or more colors [5].

11. Conclusion

Immunofluorescence testing is invaluable in confirming a diagnosis that is suspected by clinical or histologic examination. Immunofluorescence is the visualization of antigens within cells using antibodies as fluorescent probes. The benefits of immunofluorescence are numerous, and the technique has proven to be a powerful tool for determining the cellular distribution of known antigens in frozen tissues or in the localization of specific DNA sequences on chromosomes. The method has achieved the status of combining high sensitivity with high resolution in the visualization of antigens and will be a major tool for many years to come, that any pathologist studying cells or molecules cannot afford to ignore.

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