Immunofluorescence in oral lesions

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INTRODUCTION

Immunobullous group of disorders is characterized by the production of antibodies that are directed against various constituents of the molecular apparatus that hold the epithelial cells together or that bind the surface of the epithelium to the underlying connective tissue. The ensuing damage produced by the interaction of these autoantibodies within the host tissue is seen clinically as a disease process often termed as immunobullous diseases.

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 light into visible light.[1] In 1941 Coons and Kaplan showed that these fluorescent dyes can be conjugated with antibodies and they are called as labeled antibodies. These labeled antibodies can be used to locate and identify antigens in the tissues.[2]

Immunofluorescence (IF) is a histochemical laboratory staining technique used for demonstrating the presence of antibodies bound to antigens in tissues or circulating body fluids.[1] 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 these potentially life-threatening disorders.[3]

The most commonly used fluorochromes are:[4]
- Fluorescein isothiocyanate (FITC) which produces apple-green color
- Tetramethylrhodamine isothiocyanate with a red color of fluorescence
- Phycoerythrin which also shows red fluorescence.

IMMUNOFLUORESCENCE TECHNIQUE AND ITS PROCEDURE

There are three basic types of IF technique:[5][6]
- Direct IF (DIF)
- Indirect IF (IIF)
- Complement IIF.
DIRECT IMMUNOFLUORESCENCE

DIF is the earliest form of immunohistochemistry. In this direct technique, the antigen is reacted directly with a fluorescein-conjugated antibody specific for the material sought within the tissue.

Procedure for direct technique
1. Wash the section in 0.01M phosphate-buffered saline (PBS) with the three changes over a period of 30 min
2. Drain off excess PBS and wipe the section with cellulose tissue. Cover the section with diluted conjugate and allow it to react for at least 30 min at room temperature
3. Drain off conjugate and wash with three changes of PBS over a period of 30 min
4. Excess PBS is drained off and the sections are mounted using PBS/glycerol solution. It is important not to maneuver the section under the coverslip since movement of this unfixed material can lead to considerable distortion
5. The edge of the coverslip is then sealed using varnish. A more permanent sealant using polyvinyl alcohol has also been used with success
6. Biopsy specimens are snap frozen. Frozen 4–6 μ sections are cut on a cryostat and placed on a glass slide
7. Primary antihuman antibodies conjugated to FITC fluorescein (anti-IgA, anti-igG, anti-IgM and anti-C3) are applied to each section
8. Each reagent is tested on a separate slide and examined under a fluorescence microscope.

INDIRECT IMMUNOFLUORESCENCE

Indirect IF (IIF) is a two-step serological technique for detecting the circulating antibodies in the body fluids. It uses serial dilution of patients serum applied to the section of suitable tissue substrate.[1] Conjugated antihuman IgG is then applied to localize the autoantibodies from the patient’s serum that are bound in the tissue substrate.

Procedure for indirect technique
1. Prepare patient serum dilution in PBS. Expose section to serum dilution for at least 30 min at room temperature
2. Wash over 30 min in PBS with three changes. Remove the excess PBS with cellulose tissue
3. Cover section with diluted antihuman Ig/fluorescent isothiocyanate conjugate. After 30 min, drain off the excess conjugate and wash over 30 min
4. Mount and seal as outlined under direct technique.

COMPLEMENT-BINDING INDIRECT TECHNIQUE

This three-step IIF procedure assesses whether circulating autoantibodies are capable of fixing complement. If present, the fluorescent staining of these complement-fixing antibodies is also beyond that are achieved by conventional “IF” method. The enhancement is due to the amplification, achieved through binding of more than one molecule of complement to each immunoglobulin and the subsequent visualization of the multiple molecules of complement.

The main use of this method is in the diagnosis of pemphigoid (herpes) gestation, in which the autoantibodies avidly fix complement but are difficult to detect using conventional techniques. This technique significantly increases the sensitivity of IIF in pemphigoid gestation.

Procedure
1. Frozen tissue sections of normal skin are prepared in the standard manner on cover slide
2. The patient's serum is diluted to 1:2 or 1:4 in PBS and incubated with the section for 30 min at 37°C
3. Washing with PBS is carried out three times, for 10 min each in the usual fashion, the section are fan-dried and incubated with a source of active complement for 30 min at 37°C
4. The source of complement is fresh human serum diluted to 1:20 in complement diluting buffer. Section is washed with PBS, fan-dried and covered with the optimal dilution of fluorescein-conjugated anti-C3 for 30 min at 37°C
5. PBS is used to wash the sections once again, they are fan-dried, mounted in buffered glycerol and examined under the fluorescence microscope.

VARIANTS OF IMMUNOFLUORESCENCE TECHNIQUE

Salt-splitting technique
To distinguish epidermolysis bullosa acquisita from other immunobullous diseases with subepithelial clefting, a special technique is performed. A sample of the patient’s perilesional skin is incubated in a concentrated salt solution; this causes the epithelium to separate from the connective tissue, forming an artificially induced bulla.[5,6]

Immunohistochemical evaluation shows deposition of IgG autoantibodies on the floor (connective tissue side) of the bulla where type VII collagen resides. This finding is in contrast to...
that of most forms of mucous membrane pemphigoid, in which the autoantibodies are usually localized to the roof of the induced blister. Shown in the Figure 1a and b.

APPLICATION

IF methods are used in monitoring the disease activity in some of the immunobullous disorders. These techniques have advanced the understanding and classification of the immunobullous disorders. The autoimmune blistering diseases may be subdivided into interepidermal and subepidermal blistering disorders on the basis of the level at which blistering occurs.

Intraepidermal blistering disease
• Intercellular fluorescence - typical of pemphigus
• Fluorescence in the nuclei of keratinocytes - generally observed in connective tissue diseases.

PEMPHIGUS VULGARIS

All forms of pemphigus are characterized by loss of cell adhesion, leading to acantholysis. This loss of adhesion results in intraepithelial blister formation. IgG autoantibodies are deposited in the oral squamous epithelium. IgG autoantibodies target desmoglein 3. DIF shows intercellular deposits of IgG and C3 and shown in the Figure 2, the latter are predominantly located in the lower layers of the epithelium in almost 100% of the cases of active disease.\(^4\)

PARANEOPLASTIC PEMPHIGUS

Paraneoplastic pemphigus (PNP) is a bullous dermatosis of severe prognosis, described by Anhalt et al. in 1990. The disease affects the skin and mucous membranes and is associated with neoplasms (Castleman’s disease, lymphomas and thymomas). It is very similar to PV, but it shows diversity of autoantigens (reactivity with desmoglein 3, desmoplakins and basement membrane zone [BMZ] antigens). DIF: similar pattern to that of PV, along with deposition of IgG and C3 in the BMZ shown in the Figure 3. One way to differentiate PNP from PV is to perform IIF. Circulating IgG anti-intercellular substance antibodies are always present in high titer. Rat bladder transitional epithelial cells are specific.\(^4\)Table 1 shows Immunofluorescence findings in epithelial bullous disorders.

Subepidermal blistering disease

Components of the BMZ are the target of immunological attack in these disorders, giving rise to separation at the dermal–epidermal junction.

BULLOUS PEMPHIGOID

Deposition of IgG, C3, or both at the BMZ is seen in bullous pemphigoid. Figure 4 Deposition of C3 with significantly higher intensity than IgG strongly favors the pemphigoid group of diseases.

EPIDERMOLYSIS BULLOSA ACQUISITA

Multiple deposits at the BMZ are pattern of deposition strongly favors epidermolysis bullosa acquisita. 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 shown in the Figure 5.

LINEAR IGA DISEASE

Linear deposition of IgA at the BMZ is characteristic of linear IGA disease shown in the Figure 6. Deposition of C3 is present less frequently and with lower intensity compared with IgA.

DERMATITIS HERPETIFORMIS

Granular deposition of IgA and C3 in the papillary dermis and along the BMZ is diagnostic of dermatitis herpetiformis and shown in the Figure 7. Deposition of IgA is present in 100% of patients when the biopsy specimen is obtained from normal-appearing perilesional tissue.

### Table 1: Immunofluorescence findings in epithelial bullous disorders

| Disease                          | IgG | IgM | IgA | C3  | Fibrin | Antigen     |
|----------------------------------|-----|-----|-----|-----|--------|-------------|
| Pemphigus vulgaris               | ++  | -   | -   | ++  | -      | Desmoglein3 |
| IgA pemphigus                    | -   | -   | ++  | -   | -      |             |
| Paraneoplastic pemphigus         | ++  | +   | ++  | +   | -      | Desmoplakin |
|                                  |     |     |     |     |        | Periplakin   |
|                                  |     |     |     |     |        | Envoplakin   |
|                                  |     |     |     |     |        | Desmoglein   |

### Table 2: Immunofluorescence findings in subepithelial bullous disorders

| Disease                          | IgG | IgM | IgA | C3  | Fibrin | Antigen     |
|----------------------------------|-----|-----|-----|-----|--------|-------------|
| Bullous pemphigoid               | ++  | -   | -   | ++  | -      | BP 180      |
|                                  |     |     |     |     |        | BP 230      |
| Mucomembrane pemphigoid          | ++  | -   | -   | ++  | -      | BP 180      |
|                                  |     |     |     |     |        | BP 230      |
| Epidermolysis bullosa acquisita  | ++  | -   | -   | ++  | +      | Collagen T  |
| Dermatitis herpetiformis         | -   | -   | ++  | +   | -      | Epidermal Trans Glutaminase |

### Table 3: Direct immunofluorescence findings in other conditions

| Disease                      | IgG | IgM | IgA | C3  | Fibrin | Antigen     |
|------------------------------|-----|-----|-----|-----|--------|-------------|
| Sle                          | ++  | +   | +   | ++  | -      | -           |
| Lichen planus                | +   | +   | +   | ++  | -      | -           |
skin. Deposition of C3 is seen in approximately half of cases. Deposition of IgG or IgM or both is less frequent and less intense.[7,8] Table 2 shows Immunofluorescence findings in subepithelial bullous disorders.

**LUPUS ERYTHEMATOSUS**

DIF testing of lesional tissue shows the deposition of one or more immunoreactants (usually IgM, IgG, or C3) at the BMZ and shown in the Figure 8. In addition, DIF testing of clinically normal skin of SLE patients often shows a similar deposition of IgG, IgM, or complement components. This finding is known as a positive lupus band test.[9]

**LICHEN PLANUS**

In certain cases, in which the clinical and histologic findings are not characteristic DIF and may be helpful. DIF 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 and shown in the Figure 9. 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.\textsuperscript{[10]} Table 3 shows Direct immunofluorescence findings in other conditions.

**CONCLUSION**

IF is a helpful diagnostic tool for identifying the various immunobullous disorder. It results in visualization of antigen within the cells of antibodies as fluorescent probe. It is advanced immunohistochemical method that detects immune deposits and considered a standard diagnostic protocol and helps in exact diagnosis and prognostic tool and will be a major tool for many years.

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**Conflicts of interest**

There are no conflicts of interest.

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