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Research Article

IMMUNOFLUORESCENCE IN MUCOCUTANEOUS DISEASES: A NARRATIVE REVIEW

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ABSTRACT

Mucocutaneous diseases may frequently present diagnostic dilemma as their diagnosis depends on their oral and cutaneous manifestations, histopathology, IHC, etc. Mucocutaneous diseases cannot be differentiated clinically and histopathologically from other disorders. Therefore the need of immuno-fluorescent studies have occurred as it 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 immune-fluorescence a powerful technique in the diagnosis of mucocutaneous diseases. Thus, immuno-fluorescence has become an essential component of today's diagnostic laboratory. The two main methods of immuno-fluorescent labelling are direct and indirect.

INTRODUCTION

Immunofluorescence is the combination of histochemical and immunologic methods to pin-point specific antigen-antibody complexes formed in tissue sections or cellular smears with the reaction of the fluorochrome labeled antibody (8). 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.(4)

Mucocutaneous disease (Muco: mucous membrane, Cutaneous: skin) are skin diseases involving mucous membrane such as oral mucous membrane, genital etc. The diagnosis of these Mucocutaneous disease requires clinico-pathological correlation and immuno-fluorescence methods that provide a useful adjunct to light microscopy. The relative simplicity and accuracy of the technique has made immunofluorescence a powerful technique in the diagnosis of autoimmune diseases. To confirm the clinical diagnosis of disease, both the direct and indirect IF test should be done to avoid any false positive or false negative results.

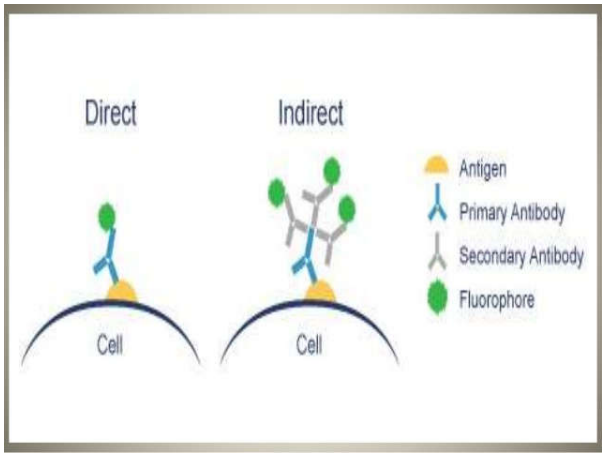

Figure 1 Direct and indirect immunofluorescence showing fluorochrome dye labelled antibodies

History

Immunofluorescence studies are considered the 'gold standard' for the diagnosis of autoimmune blistering diseases.[4] 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[4]. Later in 1954, Wells and Coons described indirect

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immunofluorescence and sandwich technique of immunofluorescence. 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.4 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. Immunoperoxidase technique, immunoelectron microscopy, immunoprecipitation studies, western blot analysis and enzyme-linked immunosorbent (ELISA) assays have been developed but IF is still widely practised.

Immunofluorescence Techniques

1. Direct technique
2. Indirect technique
    - Variants of indirect technique
    - Salt Split Technique
    - Antigenic Mapping Method
    - Double Staining Method
    - Calcium Enhancement Indirect Technique
3. Complement binding indirect technique

Direct Immunofluorescence

Direct immunofluorescence (DIF) is a one-step procedure used to detect and localize immunoreactants deposited in vivo in the patient's skin or mucosa. The immunoreactants include antibodies, complement components and fibrinogen. Frozen sections 5µm in thickness are cut with the cryotome and placed on slides. These are dried for ten minutes with an electric fan. Inadequate drying of sections between processing steps may lead to their detachment during washing. The sections are then washed in Phosphate Buffer Saline (PBS) at a pH of 7.4 for ten minutes. The sections are fan-dried once more and incubated with monospecific fluorescein isothiocyanate (FITC)-labelled antisera for thirty minutes at 37° C. Antisera to IgG, IgA, IgM, fibrinogen and the C3 component of complement should be routinely employed. Antisera to particular subclasses of immunoglobulins and other components of complement are also available but are less commonly used. Sensitivity and specificity of staining may be maximized by the use of the optimal dilution of the labelled antisera. This is determined by a chess-board titration procedure utilizing a known positive tissue specimen. The sections are washed in PBS to remove unbound antisera, fan-dried and mounted in a drop of buffered glycerol. They are then viewed with the fluorescence microscope.[11]

The distribution and type of immunoreactant deposition is recorded. The class and subclass of immunoglobulins and the presence or absence of complement is noted. Excessive fibrin depostion indicates that immunoreactants have been present more than 24-48 hours. Immunoreactants are deposited in two main patterns: in the epidermal intercellular space (ICS) and along the basement membrane zone (BMZ). Intercellular space immunoreactants may be found throughout the epidermis or restricted to certain layers. Basement membrane zone deposits may be smooth and linear, granular and discontinuous or a combination of the two.

A number of artefacts must be differentiated from significant findings. Autofluorescence by proteins such as keratin and elastic fibres may be a significant problem. Non-specific staining results from binding of eosinophils. Undesired specific staining is produced by antibodies with unwanted specificities, leading to binding of the fluorescein conjugate to irrelevant structures. A trained observer with knowledge of dermatopathology and immunofluorescence will gain considerable assistance from simultaneous examination of a haematoxylin and eosin stained frozen section to aid in identification of fluorescing structures.[11]

Indirect Immunofluorescence

Indirect immunofluorescence (IIF) is a two-step procedure used to identify circulating autoantibodies to cutaneous or mucosal stuctures in a patient's serum. These antibodies are most commonly of lgG or lgA classes.

In the first step, serial dilutions of the patient's serum in PBS are incubated with frozen sections of the substrate. At least two 5µm thick sections are prepared by being alternately fan-dried, washed in PBS and fan-dried for ten minutes each. The initial serum dilution to 1:10 or 1:80 is incubated with the sections for thirty minutes at 37° C. If positive, subsequent incubations are used with increasingly higher dilutions of sera. Autoantibodies in the serum bind to components of the epidermis and basement membrane zone. Three washings of ten minutes each in PBS are carried out to remove unbound serum.[11, 28]

In the second step, the bound autoantibodies are labelled with fluorescein isothiocyanate (FITC)-conjugated anti-human immunoglobulins. Class-specific antibodies are routinely used but subclass-specific antibodies are also avaible. Incubation with the antisera for thirty minutes at 37° C is followed by three washings in PBS of ten minutes each. The sections are then mounted in a drop of buffered glycerol and viewed with the fluorescence microscope. [11]

Many different epithelial substrates have been used for indirect immunofluorescence, however skin from the scalp, face, sun damaged sites and neonatal prepuce has been shown to yield a high rate of false-negative results. Guinea pig lip and oesophagus are also unsatisfactory for standard IIF due to a high rate of false-negatives. The best substrate to be normal human skin from non-sun exposed sites on the trunk and the flexor surfaces of the limbs. This may be obtained from breast reductions, abdominoplasties and other cosmetic procedures. The skin is transproted to the laboratory in normal saline, sectioned and then snap-frozen in OTC compound with the same technique described earlier for skin biopsies. However, certain animal epithelial substrates may have special applications for the specific diagnosis of paraneoplastic pemphigus and bullous pemphigoid.[11]

Every series of indirect immunofluorescence studies must include known positive and negative controls. For the negative controls sections are incubated with normal human serum and with FITC-conjugated anti-human lgG. The later may demonstrate non-specific and undesired specific binding of the conjugate.

The class or subclass of immunoglobulins, the pattern and the site of deposition are noted. Circulating antibodies are most commonly IgG, IgA is present in certain disorders while immunoglobulins of other classes are less common.

Immunoglobulins may bind in a linear or discontinous manner either along the BMZ or in the ICS.[11]

Salt split skin technique

The split-skin techniques have applications in the diagnosis of the immunobullous disorders with separation at the dermal-epidermal junction. Various methods exist to split skin through the lamina lucida, this being the area of least resistance in the basement membrane zone. These methods include suction blister creation or incubation in sodium chloride, trypsin and PBS. Cleavage through the lamina lucida places the hemidesmosomes and upper lamina lucida in the roof of the split and the lower lamina lucida and the sub-lamina densa in the floor.[11]

The incubation of skin in 1M normal saline for 72 hours at 4°C more reliably induces cleavage through the lamina lucida than the other splitting techniques. This technique may be used with biopsies of the patient's skin for direct immunofluorescence or skin substrate for indirect immunofluorescence.[11]

Salt-splitting the patient's skin prior to direct immunofluorescence localizes basement membrane zone immunoreactants to the roof, floor or both locations. This has applications in distinguishing between the subepidermal blistering disorders. The roof or epidermal pattern is found when binding of immunoreactants is to hemidesmosomal and upper lamina lucida antigens. Immunoreactants in the lower lamina lucida and sublamina densa produce a floor or dermal pattern. The split skin technique also increases the sensitivity of detection of BMZ immunoreactants. [11]

Antigenic mapping method

This method is used as an adjunct to electron microscopy to differentiate two major forms of epidermolysis bullosa. A blister is induced by rubbing the skin with a pencil eraser for one minute and biopsy is taken from that area after ten minutes. Biopsy material is snap frozen and slides are prepared. IIF is performed with polyclonal/monoclonal primary antibodies directed against different antigenic components of dermo-epidermal junction, including BPA, Laminin & Collagen Type

Cleavage plane is determined by noting Ag detected on floor & roof of mechanically induced blister.[28]

All the three antigens are seen on floor (Intraepithelial Split) in superficial blister, bullous pemphigoid and laminin antigen is seen on the roof and type IV collagen on the floor (intralamina lucida split) in junctional blisters, and all the three antigens are detected on the roof (sublamina densa split) in dystrophic blisters.[28]

Double staining method

In this method FITC & TRITC are used in conjunction to demonstrates co-distribution of two Ag in tissues. Double staining can be used as direct / indirect method. This indirect method has very high sensitivity.[28]

Calcium enhancement indirect technique

The sensitivity of indirect immunofluorescence in pemphigus vulgaris and foliaceus may be increased by tris-acetate buffered saline (TAS) with added calcium chloride. In this technique, TAS with 5mM CaCl2 is used to dilute patients' sera. The mechanism of this heightened sensitivity is unknown. It may occur through stabilization of calcium sensitive epitopes in the target antigens or associated proteins; facilitation of antibody binding; or protecting antigens from proteolysis. This technique will not enhance detection of antibasement zone antibodies in bullous pemphigoid or epidermolysis bullosa acquisita.[11]

Complement-binding indirect method

This three-step indirect immunofluorescence procedure assesses whether circulating autoantibodies are capable of fixing complement. If present, the fluorescent staining of these complement fixing antibodies is also enhanced beyond that achieved by conventional immunofluorescence methods. 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) gestationis in which the autoantibodies avidly fix complement but are difficult to detect using conventional indirect techniques. This technique significantly increases the sensitivity of indirect immunofluorescence in pemphigoid gestationis.[11]

Frozen tissue sections of normal skin are prepared in the standard manner on cover slides. The patient's serum is diluted to 1:2 or 1:4 in PBS and incubated with the sections for thirty minutes at 37° C. Washing with PBS is carried out three times for ten minutes each in the usual fashion, the section are fan-dried and incubated with a source of active complement for thirty minutes at 37° C. The source of complement is fresh human serum diluted to 1:20 in complement diluting buffer. Sections are washed with PBS, fan-dried and covered with the optimal dilution of fluorescein conjugated anti-C3 for thirty minutes at 37° C. PBS is used to wash the sections once again, they are fan-dried, mounted in buffered glycerol and examined with the fluorescence microscope.[11]

Sandwich Technique

Here the tissue specimen is treated with the prepared serum where the antigen will attach to the antibodies present in the specimens. Then it is treated with the flurochrome labelled antibody which will specifically go and attach to the antigens. That is the antigen which izs sandwiched between the two antibodies.[28]

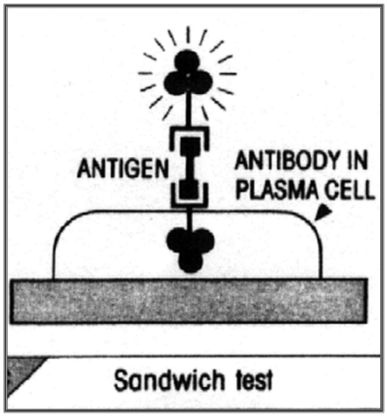

Figure 2 Sandwich Technique showing antigen and antibody reaction via fluorochrome.[28]

Appearances of Various Oral Mucosal Lesions

Mucocutaneous diseases which are caused by pathogenic autoantibodies directed against antigens in the intercellular substance of stratified squamous epithelium or dermo-epidermal junction constitutes the important group of dermatologic disorders.[4]

These can be further subdivided into intraepidermal and subepidermal depending upon level of split. Intraepidermal immunobullous diseases include the pemphigus group and its variants e.g. pemphigus vulgaris (PV) and pemphigus vegetans, pemphigus foliaceus (PF), endemic pemphigus foliaceus, pemphigus erythematosus (PE), drug-induced pemphigus, IgA pemphigus, and paraneoplastic pemphigus (PNP). Subepidermal immunobullous diseases include bullous pemphigoid (BP), pemphigoid gestationis (PG), cicatricial pemphigoid (CP), linear IgA bullous disease (LABD), epidermolysis bullosa acquisita (EBA) and bullous lupus erythematosus.

There are various targeted proteins against which autoantibodies are secreted as depicted in figure 3 and figure 4. This plays a significant role in diagnosing various mucocutaneous diseases

The main reason for the continued identification of new bullous diseases is that the diagnosis of bullous diseases at present is based on immunologic and molecular findings rather than clinical or histologic findings alone.[77]

Bullous disease	Targeted molecule
BP	BP 180, BP 230 (hemidesmosome and lamina lucida)
HG	BP 180, BP 230 (hemidesmosome and lamina lucida)
CP	BP 180, laminin V (hemidesmo-some and lamina lucida)
EBA	Type VII collagen (anchoring fibrils)
Bullous SLE	Type VII collagen (anchoring fibrils)
LAD (adults and children)	LAD antigen (BP 180) (hemidesmosome and lamina lucida)
DH	Unknown

Figure 3 Molecular classification of Subepidermal Bullous Diseases.[77]

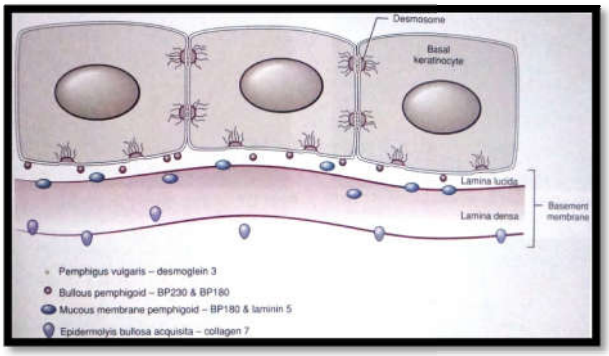

Figure 4 Photograph showing antigenic targets in various bullous disorders.[78]

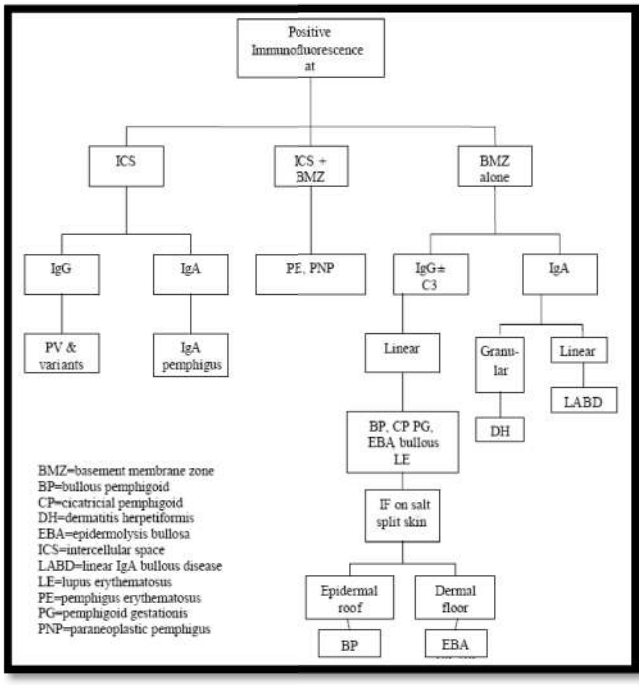

Figure 5 Algorithmic approach in diagnosing mucocutaneous diseases by Immunofluorescence.[4]

Intrae Epidermal Blistering Diseases

Pemphigus

It refers to a group of autoimmune blistering diseases of the skin and mucous membrane characterized histologically by intradermal blisters and immuno- pathologically by the finding of in vivo bound and circulating IgG antibody directed against the cell surface of keratinocytes.[5] The three primary subsets of pemphigus include:-

1. Pemphigus vulgaris (PV)
2. Pemphigus foliaceous (PF)
3. Paraneoplastic pemphigus (PNP)

Pemphigus vulgaris

Pemphigus vulgaris is characterized by thinwalled, flaccid bullae which rapidly rupture to leave slowhealing erosions. The site of onset is frequently in the mouth and other mucosae may also be involved. Biopsy findings are of separation at the suprabasilar level with acantholysis. Perilesional and clinically unaffected skin and mucosae has intercellular antibodies deposited throughout the epidermal intercellular substance (ICS) of almost all patients with active pemphigus.

Immunofluorescence has proven to be of great value in establishing the diagnosis of pemphigus, especially when the clinical or microscopic findings are inconclusive. In this test, direct immunofluorescence is used to demonstrate the presence of' immunoglobulins predominantly IgG but sometimes in combination with C3, IgA and IgM in the intercellular spaces.[5] Sano SM *et al* in 2008 considered a positive pattern for pemphigus vulgaris when IgM, IgG, IgA or C3 in the intercellular space was deposited around the keratinocyte cell surface resulting in a honeycomb pattern.[71] Daniels and Quadra-White have reported positive direct immunofluorescence predominantly for IgG followed by C3, IgA and IgM in pemphigus vulgaris patients.[80] Laskaris also

found the similar results with pemphigus vulgaris patients. By indirect immunofluorescence, Laskaris also reported that, when normal human oral mucosa was used as the substrate, circulating intercellular substance antibodies were present in majority of patients.[81] Costa *et al* in 1981 reported intercellular deposition of IgG on cytological smears from oral pemphigus lesions.[24]

Pemphigus foliaceous

Pemphigus foliaceous (PF) is generally a benign variety of pemphigus. It is an autoimmune skin disorder characterised by loss of intercellular adhesion of keratinocytes in the upper part of the epidermis (acantholysis), resulting in the formation of superficial blisters.[5] It includes following six subtypes-

1. Pemphigus Erythematosus
2. Endemic pemphigus foliaceus
3. Pemphigus Herpetiformis
4. Drug Induced Pemphigus
5. IgA Pemphigus
6. Paraneoplastic pemphigus foliaceus

Paraneoplastic pemphigus

Paraneoplastic pemphigus is a specific paraneoplastic disorder, most commonly associated with lymphomas. All mucosal surfaces may be affected by painful erosions. Cutaneous findings are highly variable and include target lesions on palms and soles as well as papules, vesicles and erosions on the trunk and limbs.Histology shows suprabasal acantholytic separation, satellite keratinocyte necrosis, basal cell vacuolation, spongiosis and epidermal exocytosis of inflammatory cell. IgG and C3 are almost invariably deposited in the intercellular substance in perilesional skin and mucosa. C3, IgG and IgM may also be deposited along the basement membrane zone.Circulating IgG antiintercellular substance antibodies are almost always present in high titre.

Subepidermal Blistering Diseases

Bullous pemphigoid (Parapemphigus)

Bullous pemphigoid is thought to be an autoimmune disease and circulating basement membrane zone antibodies have been found by the indirect immunofluorescence technique in about 80% of patients, as reported by Laskaris and Nicolis.[81] Direct immunofluorescent studies of these same patients revealed tissue-bound anti basement membrane zone antibodies of the IgG class in all oral mucosal biopsies (100%) of patients who had both mucosal and skin lesions, but in only 80% of oral mucosal biopsies in the group of patients who had only skin lesions.[81]

DIF on salt-split specimen shows epidermal mapping in about 50% of cases whereas it is combined in the rest. C3 is of less diagnostic significance. DIF is less sensitive in localized pemphigoid than generalized type.[87] Goel *et al* in 2003 studied immunofluorescence in non bullous variant variant of Bullous pemphigoid and confirmed that on direct immunofluorescence, strong IgG and C3 deposits at the dermoepidermal junction were seen. Salt split skin sections showed localization of immunoreactants to the roof.[88]

Cicatricial Pemphigoid

Mucosal and mucocutaneous junctional involvement is prominent with painful, recurring and indolent blisters that heal with scarring and adhesions, complications of which include blindness and upper are digestive tract strictures. Cutaneous lesions occur in one fourth of cases and may heal with or without scarring. A subepidermal blister is found on biopsy and there is an evolving cellular infiltrate with phases of neutrophil, eosinophil and lymphocyte predominance.

Perilesional skin and mucosae have linear deposition of IgG and C3 along the BMZ in the great majority of active cases. IgA and IgM are deposited less often.[41],[42] Deposition of immunoreactants along the BMZ of mucosal mucous glands appears to be a specific finding in cicatricial pemphigoid. Circulating IgG and IgA antibodies are usually of low titre and are detected in 30% with standard IIF methods but this may be increased to 80% by the use of saltsplit skin substrate. IgM antibodies may also be found. The binding of antibodies is most commonly to the epidermal aspect of saltsplit skin but may be to both sides or to the dermal side alone. IgG antibodies are usually IgGl and IgG4 while IgA antibodies are always of IgA subclass. Titres do not relate to disease extent nor activity.

Epidermolysis bullosa acquista

Epidermolysis bullosa acquisita tends to begin with an inflammatory phase which may mimic bullous or cicatricial pemphigoid or dermatitis herpetiformis. The classic noninflammatory mechanobullous pattern of disease may follow or be the initial manifestation of disease. Minor trauma causes ulceration which heals with milia, scarring and hyperpigmentation. Mucosal involvement is common. A cellpoor subepidermal split with variable dermal cellular infiltration is seen on biopsy. IgG is deposited linearly along the BMZ of perilesional skin in all active cases. IgA, IgM and C3 are also often present. Saltsplitting DIF techniques reveal a dermal pattern of immunoreactant deposition in all cases . This floor pattern may rarely occur in BP and therefore does not reliably differentiate EBA from BP. The detection and titre of circulating IgG antiBMZ antibodies may be increased from 25-50% using standard IIF methods to 50-85% with saltsplit skin substrate.A dermal pattern of binding is always obtained but is not entirely specific to EBA. IIF studies performed using toad skin which contains the BP antigens but not those of EBA will be negative. Circulating antibodies are more often found in the early inflammatory phase but the titre does not appear to otherwise correlate with disease activity.

Bullous Systemic Lupus Erythematosus (BSLE)

Bullous systemic lupus erythematosus usually presents with a generalized eruption of tense vesicles and bullae with a noninflamed base but may simulate bullous pemphigoid or dermatitis herpetiformis. By definition, all patients should satisfy the American Rheumatological Association criteria for diagnosis of SLE. The biopsy findings are similar to those of dermatitis herpetiformis with subepidermal separation and neutrophil papillary microabscesses.Basal cell vacuolation, Civatte bodies and dermal vasculitis are occasional findings. Direct immunofluorescence of perilesional skin shows IgG, IgM, IgA and C3 deposited along the dermoepidermal junction,

in the upper dermis and occasionally in small dermal venules. IgG is always present and IgA and IgM are also frequently deposited. The pattern of deposition may be granular (60%), linear (40%) or rarely fibrillar.

A linear rather than granular pattern along the BMZ is associated with the presence and higher titre of circulating autoantibodies. Bullous SLE is associated with a higher incidence of IgA deposition (76%) than other forms of SLE (17%) and this may also correlate with renal involvement. C3 is usually deposited in lesional skin. The circulating antibodies are usually of low titre and the sensitivity of detection is increased with the use of saltsplit skin substrate. A dermal pattern is obtained with this technique. Immunoblotting studies may sometimes be positive when saltsplit skin IIF is negative.
A high antinuclear factor titre may obscure positive BMZ fluorescence but removal of nuclear antigens from the substrate will permit its demonstration. Bullous SLE may be divided into types I and II on the basis of the presence or absence of antibodies to type VII collagen. Failure to demonstrate these antibodies with both IIF and direct immunoelectron microscopy permits classification as type II BSLE.

Linear IgA DISEASE (LAD)

Linear IgA disease (LAD) is a chronic autoimmune disease of the skin that commonly affects mucous membranes, including gingiva. It is characterised by annular to targetoid lesions with the subsequent development of the classic 'cluster of jewels' lesions of grouped small blisters around the edge of an erythematous annular lesion. Microscopically separation at the basement membrane is seen. Neutrophils and eosinophils often fill the separation.[78] In the lamina lucida type LAD, the antigens against which the IgA is directed include a 97-kD and a 120 kD protein that may be found in epidermal and dermal extracts. Both of them are part of the shed ectodomain of the 180 kD BP Ag2. In the sublamina densa type, antigens in many instances is unknown. In some cases antigen is type VII collagen, specifically the NC-I domain, the immunodominant epitope for the EBA.[27]

By definition, IgA is deposited as a linear band along the BMZ in all patients but initial false negatives may occur. Regional variation appears to exist with the forearm and conjunctiva being more often falsely negative. Other immunoreactants (IgM, IgG and C3) are also present in 20- 30% of patients. The IgA deposits in LAD lack J chains and are of the IgA1 subclass. Epidermal, dermal and combined patterns of IgA deposition may be seen with saltsplitting of the biopsy.

CONCLUSION

Direct immunofluorescent staining and subsequent microscopy is a useful tool in establishing the exact diagnosis of mucocutaneous disorders. However, in certain instances, especially in cases of subepidermal blistering lesions it needs to be reinforced with either immune electron microscopy or salt split skin technique and subsequent immunofluorescent microscopy in exactly localizing the site of immunodeposits. It also has a prognostic significance, where we feel treatment should be continued until both direct and indirect IMF are negative. So, these rapid and reliable techniques permit early diagnosis and treatment of these potentially life-threatening disorders. They are also useful in the subsequent monitoring of disease activity in some disorders. 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.

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