Diagnostic Immunopathology in 21ST Century Dermatology

Part I: Basics and Epidermal Deposits

Maria Jasmin J. Jamora

Immunofluorescence techniques and other immunopathologic diagnostic techniques in dermatology involve the detection and localization of specific autoantibodies against various antigens within the skin. Since the 1960s when the lupus band test was utilized to diagnose lupus erythematosus, diagnostic immunopathologic techniques, when interpreted alongside clinical and histopathologic findings, have become a part of the diagnostic criteria in the diagnosis of many skin diseases, in particular the autoimmune blistering diseases and connective tissue diseases. This article gives an overview of basic and updated immunopathologic techniques used in the diagnosis of many dermatologic conditions, based on the pattern of immunofluorescence staining: epidermal intercellular staining encompasses the pemphigus group and its variants, while keratinocyte cell surface staining with dermoepidermal junction staining leads to the diagnosis of paraneoplastic pemphigus. Staining of the basement membrane zone points to the diagnosis of the subepidermal blistering diseases, including the pemphigoid group and variants, epidermolysis bullosa acquisita, linear IgA bullous dermatosis or dermatitis herpetiformis. Shaggy fibrinogen staining at the basement membrane zone with cytidy bodies is characteristic of lichen planus. Vascular staining may help diagnose immune complex vasculitis, and homogeneous vascular staining combined with dermoepidermal staining suggests the diagnosis of porphyria cutanea tarda. The significance of other patterns of staining like cytidy bodies (found in interface dermatitis like lichen planus and lupus erythematosus), and anti-nuclear fluorescence (seen in mixed connective tissue disease) are also discussed. Clinical features, common immunofluorescence findings and adjunctive immunopathologic techniques like immunoblotting are detailed. The advancement of current knowledge at the molecular level is rapid, leading to the precise identification of target antigens, and production of recombinant proteins used in enzyme-linked immunosorbent assays (ELISA), which are highly sensitive and specific tests that calls into question the relevance of more traditional immunofluorescence techniques. (Dermatol Sinica 26: 119-137, 2008)

Key words: Immunofluorescence, ELISA, Immunoblotting, Pemphigus, Pemphigoid

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INTRODUCTION

Immunofluorescence (IF) techniques and other immunopathologic diagnostic techniques in dermatology involve the detection and localization of specific autoantibodies against various antigens within the skin. Initial techniques using labeling with azo-dyes or radioiodine were too weak to detect the antibodies, however this was overcome when Coons in 1942 introduced the use of fluorochromes as labels, resulting in fluorescein isothiocyanate conjugated anti-human antibodies. Initial clinical application in dermatology occurred later in 1963, when Burnham et al defined the “lupus band” as granular deposition of IgG and C3 along the dermoepidermal junction to aid in the diagnosis of lupus erythematosus then in 1964, when Beutner and Jordon used IF to diagnose pemphigus vulgaris. Since that time, immunofluorescence techniques, when interpreted alongside clinical and histopathologic findings, have become a part of the diagnostic criteria in the diagnosis of many skin diseases, in particular the autoimmune blistering diseases (AIBD) and connective tissue diseases like lupus erythematosus and vasculitides. Table 1 describes basic immunofluorescence techniques.

The basis of most immunofluorescence techniques involves the application of specific anti-human antibody to a microscopical preparation to demonstrate cellular antigens. The patient’s autoantibodies are labeled with either fluorescein or rhodamine fluorescent dyes, and when combined with the skin anti-
gen, these antigen-antibody complexes can be visualized using a fluorescence microscope. Fig. 1 illustrates some common immunofluorescence techniques. These older techniques such as direct immunofluorescence (DIF) and indirect immunofluorescence (IIF) although relatively sensitive, specific and reproducible, still have several limitations: degradation of fluorochromes over time, lack of permanence of preparations, poor resolution of IF preparations under higher magnification, expensive equipment, labor and time intensive, tissue autofluorescence, inability to use fixed or embedded tissue, and dependence of the technique on operators experience and skill.

Autoimmune skin diseases are diseases associated with an immune response directed to self-antigens, autoreactive T-cells and B-cells, and autoantibodies that manifest disease. Starting at the end of the 20th century, immunologic diagnostic techniques have elucidated the structures in blistering diseases mediating skin adhesion, such as the keratinocyte cytoskeleton, the desmosome, hemidesmosome, extracellular matrix proteins and all other proteins relevant to autoimmune blistering diseases. The advancement of knowledge at this molecular level, identification of target antigens, and production of development recombinant proteins have led to new techniques that detect specific autoantibodies, such as the enzyme-linked immunosorbent assay (ELISA), which are proving to be highly sensitive and specific, and calls into question the relevance of more traditional IF techniques.

The format of this review of diagnostic immunopathologic techniques in current dermatology will introduce diseases based on the predominant immunofluorescence staining pattern. Diagnostic algorithms of

![Fig. 1](image)

Different immunofluorescence techniques. (A) Indirect immunofluorescence using monkey esophagus substrate; linear deposition of C3 on the basement membrane zone (BMZ) in a patient with bullous pemphigoid (BP). (B) Indirect immunofluorescence using human skin as substrate; linear deposition of C3 at the BMZ in a BP patient. (C) Salt split skin using normal human skin and incubated with epidermolysis bullosa acquisita (EBA) patient’s sera, show linear staining of IgG at the dermal (floor) side of the blister in a case of EBA. (D) Salt split skin using normal human skin incubated in 1M NaCl solution and incubated with BP patient’s sera shows linear staining at the epidermal (roof) side of the blister in a case of BP.
Table 1 Basic Immunofluorescence Techniques Used in Diagnostic Immunopathology, with Definition and Brief Description of Technique

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<th>TECHNIQUE</th>
<th>DEFINITION AND DESCRIPTION</th>
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<td>DIRECT IMMUNOFLUORESCENCE (DIF)</td>
<td>Direct immunofluorescence (DIF) involves the application of fluorescein conjugated antibodies to frozen sections of skin. It identifies the location, pattern of staining, intensity and types of immunoreactants deposited in the skin. Common panel of immunoreactants include IgG, C3, IgM, IgA, and fibrinogen. In DIF, fluorochrome-conjugated antibody is added directly to the patient’s tissue, which then binds to the antigen-antibody complexes in the skin. Indirect immunofluorescence (IIF) involves identifying antibody in patient serum by incubating with a tissue substrate with a known antigen, then incubating with a specific fluorochrome-labeled secondary antibody directed against the patient’s autoantibodies. Variations on immunofluorescence techniques include the labeled antigen method, the protein A conjugate method and the avidin-biotin method.</td>
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<td>SALT SPLIT SKIN</td>
<td>Human skin may be incubated for 24 hours with 1M NaCl causing a split at the lamina lucida, the weakest part of the basement membrane zone. In both DIF (using patients own skin) or IIF (using normal human skin and reacting with patient serum), it may identify immunoreactant staining on the epidermal side (“roof”) of the blister, suggesting the diagnosis of bullous pemphigoid (BP) and certain types of cicatricial pemphigoid (CP) and linear IgA bullous dermatosis, whereas staining on the dermal side (“floor”) of the blister suggests the diagnosis of epidermolysis bullosa acquisita (EBA), anti-epiligrin cicatricial pemphigoid or bullous lupus erythematosus.</td>
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<td>INDIRECT IMMUNOFLUORESCENCE (IIF)</td>
<td>Indirect immunofluorescence (IIF) is a semi-quantitative assay that detects circulating antibodies in patients with various bullous diseases, including pemphigus. It involves applying patient serum containing autoantibodies to normal whole tissue substrate, which may include human skin, monkey or guinea pig esophagus, and then applying fluoresceinated antibodies. It has been the standard for detecting circulating antibodies, and is better than the ELISA for the initial evaluation of patients with blistering diseases, as it permits the simultaneous evaluation of antibodies associated with multiple autoimmune bullous diseases. Serially diluted patient sera is reacted with various substrates and stained with fluorescein-conjugated anti-human IgG antibodies. The sensitivity of IIF increases when using 2 substrates rather than just one. In pemphigus, IIF allows the estimation of antibody levels which roughly parallels disease activity and may help guide therapy. However, IIF has some disadvantages: 1) the subjective technique relies on examiners experience 2) discontinuous titer values make it only semi-quantitative.</td>
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<td>ANTIGEN MAPPING</td>
<td>Antigen mapping is based on the separation of the basement membrane (BMZ) above the lamina densa and mapping the location of 3 BMZ components in the patient’s blister: BPAg, laminin and type IV collagen.</td>
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<td>COMPLEMENT FIXATION</td>
<td>Complement fixation is a type of IIF, which involves layering patient serum on substrate, then adding complement. Fluorescein anti-complement antibodies are used to detect the presence of complement in tissue. This test can detect small amounts of antibodies, since a single antibody can fix many molecules of complement, thus amplifying the signal. It is commonly used to detect HG (herpes gestationis) factor. Salt split human skin is incubated with patients serum, washed, treated with human serum as source of complement, which is then visualized using fluorescein isothiocyanate-conjugated goat anti-human C3 antibody.</td>
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IMMUNOBLOTTING (WESTERN BLOTTING) Immunoblotting, or Western blotting, identifies the presence of circulating antibodies as well as the specific individual antigens against which they are directed. It involves extracting proteins from human epidermis, performing gel electrophoresis and transferring proteins onto a nitrocellulose membrane. Serially diluted patient sera are then incubated and stained with peroxidase-conjugated anti-human IgG, and either chemically or radiographically visualized. When positive, immunoblotting can establish the diagnosis for many immunobullous diseases. Disadvantages however include: 1) lack of reactivity due to protein degradation and destruction of epitopes; 2) difficult, time-consuming procedure; 3) only qualitative; 4) impractical for screening large numbers of samples. Since normal human epidermis is used as the source of antigen for the immunoblotting substrate, variations in antigen density may occur.

IMMUNOPRECIPITATION TECHNIQUE Serum IgG fractions are isolated using 40% ammonium sulfate, followed by dialysis in PBS, lyophilized and reconstituted in PBS. Cultured keratinocyte monolayers are metabolically labeled in calcium labeling medium, and S-methionine labeled proteins are separated by centrifugation and used as a source of keratinocyte proteins in standard immunoprecipitation assay. Immune complexes are precipitated with protein A-agarose suspension, washed and resolved on SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). Gels are fixed and enhanced, and radioactivity analyzed using storage phosphor autoradiography and analyzed for specific immunoprecipitation.

IMMUNOELECTRON MICROSCOPY (IEM) Immunoelectron microscopy (IEM) is conducted similarly to DIF and IIF. Preblistered skin is cut 14 microns thick and antibodies are labeled with an enzyme (either horseradish peroxidase or colloidal gold), and visualized using an electron microscope. IEM visualizes the subcellular localization of immunoreactants and is probably the most definitive tool to differentiate BP from CP and pemphigoid group from EBA. IEM may be done directly or indirectly using patient’s sera, using pre- or post-embedding technique double immunogold labeling technique.

ENZYMELINKED IMMUNOSORBENT ASSAY (ELISA) In ELISA systems, recombinant proteins (antigen in the substrate) representing the ectodomain of the target antigen are placed in plates, then incubated with patients serum which binds to the substrate. Plates are washed then incubated with mouse monoclonal anti-human antibodies. Chemical produces a color change upon reaction with previously formed complexes, and a stop solution applied. Binding of complexes is detected by a series of amplification steps that produce visible fluorescence or colored dye, which is detected and quantified by an automated machine photometer as optical density. Color change is related to the amount of antibodies present in the sample. ELISA score is then presented as an index value, which is a ratio of the absorbance of the tested serum to a positive control serum. In general, the ELISA has proven to be useful in the diagnosis of both pemphigus and bullous pemphigoid, having a higher sensitivity overall than IIF using 2 substrates. It can be used as a confirmatory test for patients who have positive IIF studies, further identifying autoantibodies targeted against specific antigens. Advantages of the ELISA in general include: a relatively simple, rapid, objective and reproducible technique that allows screening of large numbers of samples, thereby allowing less expense and labor; antigen specificity, quantitation of antibody levels giving continuous data, and making it useful to monitor disease activity. Disadvantages include: difficulty in using as a screening tool due to antigen specificity; unexpected results in patients undergoing therapy or remission; false positive results in patients with other immunobullous diseases. Therefore, DIF and IIF must be used initially as screening prior to using ELISA testing.
skin diseases based on IF staining patterns will be demonstrated (Fig. 2). Each section will have a brief clinical overview of the disease, a discussion of the identified target antigen and pathogenic antibodies, immunofluorescence findings, and finally advances in antibody detection using ELISA and other immunopathologic techniques. Excellent reviews of immunofluorescence techniques, diagnostic immunopathology and updates on molecular mechanisms in autoimmune skin diseases may be found in other articles.5, 10, 21, 23, 24

I. EPIDERMAL INTERCELLULAR DEPOSITS: PEMPHIGUS GROUP

Intercellular keratinocyte staining of immunoglobulins (IgG and IgA) as well as complement are characteristic of the pemphigus group and its variants (Fig. 3). Pemphigus is an autoimmune blistering disease affecting mucous membranes and skin, and is mediated by pathogenic autoantibodies against the keratinocyte cell surface antigen desmoglein (Dsg), a desmosomal cell adhesion molecule.25

It is subdivided into 2 major subgroups: pemphigus vulgaris (PV) and its main clinical variant pemphigus vegetans, and pemphigus foliaceus (PF) including endemic Brazilian fogo selvagem (FS), and its major variant pemphigus erythematosus (PE). Until recently, the detection of pemphigus antibodies relied on established immunologic tests such as DIF, IIF, and immunoblotting (IB) or immunoprecipitation (IP).7

1. Pemphigus vulgaris and variants (pemphigus vegetans)

PV is a deeper form of pemphigus that presents clinically with flaccid vesicles, bullae and erosions distributed predominantly

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**Fig. 2**
Algorithm for diagnosis of epidermal intercellular deposits by patterns of staining on immunofluorescence.
on flexural areas and commonly affecting the oral mucosa. Histopathology shows suprabasal acantholysis, and DIF reveals intercellular deposits of IgG. IIF on various substrates will also show a reticulated or net-like pattern of intercellular deposition of IgG.

Pemphigus vegetans is a rare variant of PV characterized by hypertrophic verrucous granulating plaques arising from pustules and flaccid bullae located on the intertriginous folds and oral mucosa. It clinically presents with 2 subtypes: the Neumann type (flaccid bullae and erosions with a course similar to PV), and Hallopeau type (more pustular form with a less severe clinical course). Histopathology will show suprabasal acantholysis within a markedly hyperplastic epidermis, and intraepidermal eosinophilic microabscesses. Pemphigus vegetans will have similar immunofluorescence findings as PV, with epithelial intercellular staining of IgG and C3 (rarely IgM, and IgA), and IIF on monkey esophagus detecting autoantibodies against desmoglein-3 (Dsg-3). Immunosuppressive therapy includes corticosteroids, cyclosporine, cyclophosphamide and etretinate.25

Majority of sera from PV patients recognize predominantly conformational epitopes of the extracellular domain of Dsg-3, a 130kDa protein, as well as desmoglein-1 (Dsg-1) in 60% of patients.7,9 Dsg is a desmosomal cadherin with 4 isoforms, Dsg-1, Dsg-2, Dsg-3, and Dsg-4.26,27 Dsg-2 is expressed in all desmosome-bearing tissue like simple epithelia and myocardium, and Dsg-4 is found in the stratum corneum and granulosa of epidermis and matrix cells of anagen hair bulb.27 Dsg-1 and Dsg-3 are restricted to stratified squamous epithelia and are the main target antigens in all forms of pemphigus. Passive transfer studies have shown that

![Image](image-url)

**Fig. 3**

Intercellular staining of IgG in a patient with pemphigus vulgaris using direct immunofluorescence. (fluorescence microscopy, original magnification x 200). (Used with permission from Antoinette F. Hood, MD and Steven D. Billings, MD)
anti-Dsg-1 and anti-Dsg-3 IgG autoantibodies in pemphigus are pathogenic and can induce blisters in skin organ culture and neonatal mice.\textsuperscript{27} It is thought that pathogenic autoantibodies in typical pemphigus inhibit the adhesive function of Dsg directly by steric hindrance through binding of antibody to target antigen, or by triggering signal transduction, activating a signaling pathway to induce proteases, leading to acantholysis.\textsuperscript{22, 28} De Bruin\textit{et al} reported an alternative pathogenesis for PV, suggesting that plakoglobin regulates and suppresses transcription of c-Myc, which when accumulated at high intranuclear levels, leads to loss of intercellular adhesion. PV IgG induces signals downstream of c-Myc that disrupt desmosomal plaques at the plasma membrane.\textsuperscript{29} Apart from Dsg-1 and Dsg-3, Grando and colleagues have identified other human molecules targeted by PV IgG autoantibodies: α9 (an acetylcholine receptor with dual, muscarinic and nicotinic effects) and pemphaxin (annexin-like molecule that also binds acetylcholine).\textsuperscript{15}

The traditional serological assay for pemphigus is IIF, which has the sensitivity to detect the intercellular antibodies associated with this disease in approximately 70-90\% in patients with active pemphigus.\textsuperscript{16} The test can differentiate PV from PF based on the stronger reaction of antibodies in PV to monkey esophagus (rich in Dsg-3) and the stronger reaction of PF antibodies to guinea pig esophagus (rich in Dsg-1)\textsuperscript{4} and by the preferential reaction of PF antibodies to the superficial layers of human skin whereas PV antibodies react preferentially to deeper epidermal levels.\textsuperscript{20} However, these improvements of IIF are not widely used because they require the use of multiple substrates (monkey or guinea pig esophagus, or human skin) and because there is an overlap between these findings. IB reveals presence of antibodies directed against Dsg-3, a 130 kDa antigen. It has a reported sensitivity of around 83\% for PV sera.\textsuperscript{8, 10}

ELISA was introduced approximately in 1999, which used recombinant Dsg-1 and Dsg-3 protein produced by a baculovirus expression system that secretes Dsg epitopes in its native conformational form. The secreted forms include the entire extracellular domains of Dsg 1 and 3, with a His tag at the carboxyl-terminal end, fused with the constant region of human IgG1.\textsuperscript{19, 30} The ELISA allows selective detection of antibodies against these Dsg epitopes, which include pathogenic antibodies in patients with pemphigus.\textsuperscript{9} Several studies have compared the ELISA with immunofluorescence and immunoblot.\textsuperscript{8-10, 14, 20, 32, 33} In most of the studies, all 3 assays correlate well with clinical and histopathologic diagnosis, as well as each other. Sensitivity for Dsg-3 ELISA for PV has ranged from 85\% to 100\% (latter value seen in untreated patients). Most of the studies concurred that the ELISA was more sensitive and specific than either immunofluorescence or immunoblot. The ELISA can be used as confirmatory test for patients who have intercellular antibodies detected by IIF, and to differentiate between PV and PF. Other advantages cited included: 1) ability to quantify levels of antibody present, and usefulness in monitoring disease activity\textsuperscript{10}; 2) fairly simple, standardized, rapid and reproducible technique, using only small amounts of serum sample at 1 dilution, thus allowing large numbers of samples to be screened in a short period of time resulting in less labor and expense; 3) collection of objective data with values obtained in a continuous scale\textsuperscript{10}; 4) ability to measure anti-Dsg 1 and 3 antibodies separately, allowing relating antibody levels not just with disease severity but with clinical phenotype\textsuperscript{7}; 5) ability to detect autoantibodies against conformationally sensitive epitopes, unlike IB, which cannot.\textsuperscript{29} In addition, because of the greater sensitivity of ELISA to IIF, the test should be used in
patients suspected to have pemphigus but whose IIF tests are negative.

In patients with undiagnosed blistering diseases, however, the ELISA can only evaluate the presence of Dsg antibodies associated with pemphigus, unlike IIF which can simultaneously test for antibodies associated with other autoimmune blistering diseases, including pemphigoid, epidermolysis bullosa acquisita (EBA) and paraneoplastic pemphigus (PNP). Other limitations of the Dsg ELISA include a lack of specificity for pemphigus, as anti-Dsg antibodies may be present in patients with bullous pemphigoid (BP), or connective tissue disease like lupus erythematosus (LE) resulting in a false positive result, and when used as a screening tool, ELISA is unable to detect antibodies found in multiple bullous diseases like pemphigoid or EBA.9, 20, 33, 36

Amagai and colleagues have shown in several studies that the clinical phenotype of classic pemphigus is defined by the autoantibody profile to Dsg. A majority of studies have correlated the clinical phenotype of pemphigus with the anti-Dsg antibody profile.19 Oral manifestations are mediated by Dsg-1 antibody levels, while skin manifestations are determined by Dsg-3 autoantibody levels.7, 34 They proposed a diagnostic algorithm using the Dsg ELISA: all Dsg-3 (+) serum, regardless of Dsg-1 reactivity, is given the diagnosis of PV.19, 20 The presence of Dsg-1 positivity in PV sera associates it with mucocutaneous disease versus purely mucosal PV.10 All Dsg-1 (+) and Dsg-3 (-) serum are diagnosed as PF. This combination of ELISA results allows the differentiation of PV and PF based on Dsg-1 and Dsg-3 reactivity.19 Few studies however have shown that there is a significant minority of PV patients whose ELISA autoantibody profile was not predictive of clinical pemphigus phenotype, suggesting that factors other than Dsg antibody profile play a role in the clinical appearance of PV.35

Mentink et al used an IgA ELISA to recombinant extracellular domains of Dsg-1 and Dsg-3, and found IgA antibodies to both Dsg-1 and Dsg-3 in patients with PV, PF, and PNP patients, with most case showing IgA specificity following IgG antigen specificity.32 In 6 patients that presented with both IgG and IgA deposition on keratinocyte cell surfaces in DIF studies, Oiso et al used ELISA using recombinant Dsg-1 and Dsg-3, and found that these patients with “IgG/IgA pemphigus” target predominantly Dsg-1.38 Hisamatsu et al developed an ELISA using recombinant proteins consisting of entire extracellular domains of human desmocollins 1-3 (Dsc 1, 2, 3) in baculovirus, and tested these for IgG or IgA antibodies in 165 sera of various autoimmune bullous disease. Only 1 atypical pemphigus sera and none of 45 typical pemphigus sera showed any autoantibodies to desmocollins, and authors suggested that baculovirus-expressed desmocollins may not adapt the correct conformation to detect autoantibodies.39

Nishifuji et al developed an ELISA that detected Dsg3-specific autoimmune memory B cells quantitatively in patients with severe PV. The detection of circulating activated memory Dsg-3 specific B cells suggested the important role of cell mediated immunity in PV, and the key role of HLA class II restricted CD4+ T cells in autoantibody production of PV.40 Further, Caproni et al investigated the upregulation of the CD40/CD40 ligand system in PV using immunohistochemistry, which showed CD40+ cells infiltrating perivascular dermis, while ELISA for soluble CD40 ligand showed significant increase in PV sera versus controls.41

2. Pemphigus foliaceus and variants

Pemphigus foliaceus (PF) is the superficial form of pemphigus that presents clinically with scaly and crusted lesions on
the upper trunk and extremities and no mucosal involvement. PF may be non-endemic or endemic (fogo selvagem, FS), which is predominant in South America (commonly Brazil) and in Tunisia and is thought to be caused by a still unidentified environmental factor that causes chronic antigenic stimulation. Histologically, a split occurs in the subcorneal level of the upper epidermis with acantholysis. The target antigen has been identified by IB to be Dsg-1, a 160 kDa antigen that is a transmembrane protein member of the cell adhesion supergene family. The intracellular domain of Dsg-1 interacts with plakoglobin (a desmosomal plaque component) which links it to the keratinocyte cytoskeleton, while the extracellular domain has relevant epitopes that mediate cell-cell adhesion. Passive transfer of anti-Dsg 1 antibodies from PF patients into neonatal mice induces clinical features of PF. In PF, IgG subclass distribution has identified IgG1 and IgG4 subclass specific antibodies to Dsg-1 and 3, and levels of both decrease with therapy. The IgG4 subclass predominates and has been identified to be pathogenic, recognize conformational epitopes, and correlate with disease activity and severity. The IgG1 subclass of pemphigus antibodies on the other hand are seen in patients constantly throughout disease course, and in patients in clinical remission, and in low levels in relatives of pemphigus patients. Although initially thought to be non-pathogenic, these IgG1 antibodies in PF patients recognize a linear epitope on the ectodomain of Dsg-1, and a conformational epitope on the N-terminus of Dsg-1 (the first 87 amino acids of Dsg-1 ectodomain). These IgG1 autoantibodies likewise react to the Dsg1 ectodomain by IB. Using a competition ELISA, IgG1 subclass antibodies were eventually demonstrated to induce experimental PF in neonatal mice as well.

DIF of PF lesions will show intercellular staining with IgG in upper epidermal layers (predominance of IgG4 subtype), and/or C3 and IgG1 deposits, with a similar rate of positivity for IgG deposits (approximately 95%) on skin taken from lesional, perilesional or uninvolved skin. Perilesional biopsies of PF skin showed higher positivity using C3 immunoreactants (40%) over lesional or uninvolved skin (34% for both). Pegas et al. suggested that taking uninvolved skin for DIF in PF patients could be helpful as lesional or perilesional skin to demonstrate pemphigus antibodies. Assays for circulating PF autoantibodies include IIF, IB and ELISA. IIF using monkey esophagus will reveal intercellular antibodies but cannot distinguish between PF and PV. ELISA using recombinant Dsg-1 and 3 are sensitive and specific for the detection of PF autoantibodies, and will reveal presence of antibodies to both Dsg-1 and 3, although anti-Dsg1 autoantibodies predominate. Sensitivity of IIF for PF/FS is 81% while sensitivity of IB is 33-45%. Sensitivity of ELISA for Dsg 1 in PF/FS 1 ranges from 90-100%, with a specificity of 97.4%. Sensitivity for ELISA to Dsg3 is 7-12%.

In FS, 95% of patients have anti-Dsg1 antibodies detected by ELISA, however, 20 to 55% of normal people living in endemic areas have circulating anti-Dsg1 antibodies, and FS prevalence rate of 3.4% decreases with physical distance from the endemic regions. These anti-Dsg1 antibodies from FS patients recognize epitopes located on the EC5 domain of Dsg, and precedes onset of clinical disease by 1-7 years. With onset of clinical disease, anti-Dsg autoantibodies shift to target epitopes in EC1 and EC2 ectodomains of Dsg1. Recent studies have also found anti-Dsg3 by ELISA in 7-43% of PF or FS patients, demonstrated by IP. These autoantibodies were pathogenic as illustrated by passive transfer experiments into neonatal
mice, but in FS patients, Dsg-3 levels are too low to produce clinical oral lesions.\(^7\) Antibodies to both Dsg 1 and 3 (speculated to be due to cross-reactivity) are detected in 43% of FS patients and in 15% of normal subjects, and autoantibodies were confirmed by IP. ELISA is therefore highly sensitive and specific and is a reliable test to identify and classify patients with all types of pemphigus, while having limited use in regions endemic to FS due to prevalence of anti-Dsg1 antibodies in healthy controls.\(^43\)

Pemphigus vulgaris may transition into pemphigus foliaceus and rarely vice versa, and this is speculated to be due to epitope spreading.\(^42\) Since anti-Dsg autoantibody profile tends to define clinical phenotype of pemphigus this change is reflected as quantitative changes in the ratio of Dsg-1 to 3 antibodies. Harman and Tsuji used ELISA to both Dsg-1 and Dsg-3 in 82 patients with PV presenting with oral (corresponding to Dsg-3 autoantibodies) and skin lesions (corresponding to Dsg-1 autoantibodies). Decline of Dsg-3 antibodies was correlated to disappearance of oral lesions, while rise is Dsg-1 autoantibody levels correlated with more severe skin involvement concluding that clinical features is a reflection of quantitative and qualitative change in antibody profile as detected using the Dsg ELISA.\(^30, 51\)

3. Pemphigus erythematous

Pemphigus erythematous is considered a variant of pemphigus foliaceus, and is diagnosed usually by a combination of features of pemphigus (scaly crusted erosions on seborrheic areas) with lupus erythematosus (butterfly malar rash, Raynauds phenomenon and photosensitivity, anemia and positive ANA and anti-dsDNA on serology). In some cases, SLE may precede onset of PF features by years.\(^43\) Histopathology shows subcorneal pustules with upper epidermal acantholytic granular cells. Target antigen is the same as PF, Dsg-1. DIF reveals immune deposition of immunoglobulins and complement in a broad linear-granular character at the dermoepidermal junction in combination with keratinocyte cell surface immunoreactant deposition in a pemphigus pattern. IIF on human skin or monkey esophagus will detect PE sera containing anti-Dsg antibodies, but may be difficult to interpret due to high background staining within keratinocyte nuclei and cytoplasm.\(^13\) IB may likewise be negative due to failure of antibodies to detect conformational epitopes destroyed during antigen extraction. ELISA testing using recombinant Dsg-1 and 3 will clearly demonstrate anti-desmoglein 1 autoantibodies, but not anti-Dsg-3 antibodies.\(^43\) Additional 230kDa (BP230) autoantibodies and 190kDa (periplakin) autoantibodies have also been described, which may account for BMZ staining on IF.\(^52\)

4. Drug-induced pemphigus

Drug-induced pemphigus may be identical to idiopathic pemphigus clinically, histologically and immunologically. Histopathology may show eosinophilic spongiosis with basal vacuolization, and epidermal acantholysis.\(^53\) IIF will show increased IgG autoantibodies to intercellular substances in monkey esophagus. Common medications inducing pemphigus include: ACE inhibitors like captopril and cilazapril, glibenclamide and rifampin. Drugs inducing pemphigus are classified according to chemical structure as either: 1) drugs containing sulfhydryl radical (thiol drugs) like penicillamine, captopril and enalapril, which are able to cause antibody-independent acantholysis directly in vivo;\(^54, 55\); and 2) non-thiol drugs which share an active amide group in molecule which may induce disease.\(^56\) Patients in the first group were more likely to show spontaneous recovery in 40-52.6% of cases, whereas patients in group 2 recovered spontaneously only 15% of the time.\(^56\) Korman et al performed IP using blis-
ter epidermal extracts on drug-induced pemphigus patients, and found sera antibodies directed against Dsg-1 and 3. ELISA tests to Dsg may be used to monitor disease progression, and Nagao et al showed in a case of penicillamine-induced pemphigus that upon withdrawal of drug resulted in a rapid decline in levels of anti-Dsg1 antibodies.

5. IgA (neutrophil-mediated) pemphigus

IgA pemphigus is a variant of pemphigus with a varied clinical and histopathologic picture, and is characterized immunopathologically by the presence of only IgA autoantibodies on the epithelial cell surfaces in skin and mucous membranes. It is further subdivided into: the subcorneal pustular dermatosis (SPD) type, and the intraepidermal neutrophilic IgA dermatosis (IEN) type. Distinguishing histologic features are presence of intraepidermal acantholysis with an intense neutrophilic infiltrate. Most patients may be treated with dapsone, colchicine, etretinate or isotretinoin, but may be rarely refractory and require more aggressive immunosuppressants such as cyclophosphamide and plasmapheresis.

IgA autoantibodies have been reported to be directed against both desmogleins and desmocollins however more specific antigens have yet to be identified. Desmocollins are a type of desmosomal cadherin with 3 isoforms, Dsc-1, Dsc-2 and Dsc-3. Hashimoto et al developed COS7 cells transfected with vectors containing coding sequences for human Dsc-1, Dsc-2, and Dsc-3. They found that all patients with SPD-type IgA pemphigus reacted with the surface of cells expressing Dsc-1, but not Dsc-2 and Dsc-3, while none of their IEN-type IgA pemphigus patients reacted with any of the desmocollins. They concluded that human Dsc-1 is an autoantigen for the SPD-type of IgA pemphigus.

On DIF, IgA deposition is variable and patchy, and located in the intercellular space of the upper epidermis. Circulating IgA autoantibodies of very low titer may also be demonstrated by IIF. A study by Hashimoto and Komai using IIF to human skin revealed anti-cell surface IgA antibodies in all 22 patients tested, and no IgG anti-cell surface antibodies. All samples from 10 patients with SPD-type IgA pemphigus reacted with the cell surface in the uppermost epidermis, while those with IEN-type IgA pemphigus showed variable reactivity to entire or lower epidermis. Standard IB showed no good results with any known epidermal component proteins, suggesting that epitopes for IgA pemphigus are conformationally dependent, and cannot be detected by IB. Likewise, immunoprecipitation cannot detect antigens for IgA antibodies.

If IgA pemphigus if suspected, it is necessary to request the evaluation of circulating IgA autoantibodies. Karpati et al utilized an IgA antibody detection modified ELISA of baculovirus expression system, which found IgA antibodies against the extracellular domain of Dsg-1 baculoprotein, but no antibodies against components of keratinocyte cell surface other than Dsg-1. Hashimoto et al also used a modified ELISA (by incubating plates with peroxidase-conjugated mouse monoclonal anti-human IgA antibody) that could detect IgA antibodies to Dsg-1 and Dsg-3. Of 22 IgA pemphigus patient serum samples reacted with either Dsg-1 or Dsg-3. Of 9 patients with IEN-type IgA pemphigus, only 1 each reacted with Dsg-1 and Dsg-3, confirming that the target antigen in the IEN-type of IgA pemphigus is heterogeneous. None of the samples from 10 patients with SPD-type IgA pemphigus reacted with either Dsg-1 or Dsg-3, confirming that antibodies in this variant react with a non-Dsg target antigen (previously identified to be desmocollin). Using the standard Dsg ELISA, none of 22 IgA pemphigus serum
samples showed anti-IgG antibodies to Dsg-1 or Dsg-3. Results were confirmed by immunoadsorption studies.  

**6. Pemphigus herpetiformis**

Pemphigus herpetiformis is a rare variant that combines clinical features of dermatitis herpetiformis (severe pruritus, symmetric vesicular eruption that presents in a herpetiform distribution, erythematous urticarial plaques, and occasional mucous membrane involvement), with histopathologic and immunopathologic features of typical pemphigus (upper epidermal acantholysis, eosinophilic spongiosis, and intraepidermal pustules on histopathology, and in vivo and circulating IgG autoantibodies).  

The mean age of onset is 65 years, and has no sex predilection. It has a benign course, is easily controlled with low dose systemic steroids, and usually remits completely. Some cases may progress to pemphigus foliaceus.  

IB and immunoprecipitation have shown that IgG autoantibodies in the majority of pemphigus herpetiformis patients target Dsg-1, as supported by the predominant staining of the upper epidermis in DIF studies and confirmed by immunoadsorption studies. It may also recognize Dsg-3. It is speculated that autoantibodies in PH cause inflammation through complement pathways or cytokine induction (keratinocyte IL-8), recruiting neutrophils to the upper epidermis or resulting in eosinophilic spongiosis. The autoantibodies inability to induce acantholysis is speculated to be due to recognition of a functionally less important part of the Dsg molecule, in contrast to classic pemphigus pathogenic autoantibodies.  

DIF will reveal in-vivo bound intercellular IgG autoantibody deposition in the upper or entire epidermis. IIF will demonstrate circulating IgG4 subclass autoantibodies. In patients with an atypical clinical course presenting with recalcitrant intraepidermal pustules, DIF and IIF studies may be invaluable and detect pemphigus herpetiformis or other autoimmune blistering disorders.  

A study was done by Ishii et al on IIF (+) serum of patients with pemphigus herpetiformis compared immublot techniques and ELISA (using recombinant extracellular domains or Dsg-1 and 3 (rDsg-1 and rDsg-3). ELISA results showed that 16/20 sera of PH patients were positive against Dsg-1 and 4/20 reacted with Dsg-3. Immunoadsorption studies excluded the possibility that antibodies would react with molecules other than Dsg-1 or Dsg-3. IgA class anti-Dsg1 and anti-Dsg3 ELISA was likewise done, with 2/17 samples reacting with each antigen, despite negative IIF studies using the IgA. The study indicated that Dsg-1 is the most frequently recognized antigen in pemphigus herpetiformis and Dsg-3 is an alternate target antigen, implying that pemphigus herpetiformis is likely a variant of either pemphigus foliaceus or vulgaris.  

**7. Paraneoplastic pemphigus**

Paraneoplastic pemphigus (PNP) is a rare autoimmune blistering disease of both skin and mucous membranes, and is strongly associated with underlying malignancies, mostly lymphoproliferative neoplasms such as Castleman’s tumor, Non-Hodgkin’s Lymphoma, and chronic lymphocytic leukemia, and rarely thymomas, epithelial origin carcinomas, mesenchymal origin sarcomas, malignant melanoma, adenocarcinoma, and squamous cell carcinoma. Initial criteria for diagnosis was defined by Anhalt et al as follows: 1) mucosal ulcerations and blisters, and polymorphic skin lesions with underlying malignant neoplasm; 2) histologic features of interface change, keratinocyte necrosis and intraepidermal acantholysis; 3)
intercellular deposition of IgG and C3 in the epidermis, and along BMZ; 4) serum autoantibodies that bind to cell surface on monkey esophagus and transitional epithelium such as rat bladder; and 5) immunoprecipitation of autoantibodies that recognized antigens 250, 230, 210, 190 and 170kDa. Patients should have satisfied 4 of 5 criteria to be diagnosed with PNP. Diagnostic criteria was later revised by Camisa and Helm and included major criteria (polymorphous cutaneous eruption, concurrent internal malignancy, and serum immunoprecipitation of a “ladder of antigens” from the plakin family, BPAG1, and desmosomal proteins), and minor criteria including histologic acantholysis and interface change, DIF showing combination of keratinocyte cell surface and BMZ IgG staining, and IIF of transitional epithelium such as rat bladder. Three major or 2 major and 2 minor criteria were required to diagnose PNP. PNP presents clinically with a severe mucositis (in particular erosive oral disease, also involving aerodigestive tract, eyes and genitalia), and a polymorphous cutaneous eruption which may range from lichenoid skin lesions resembling lichen planus, targetoid lesions resembling erythema multiforme and vesicular lesions resembling pemphigus. Complications include pulmonary involvement (bronchiolitis obliterans and respiratory failure that may result in death), found to be due to necrotic cellular debris occluding distal bronchi. PNP usually responds poorly to therapy, although excision of malignant tumors may result in alleviation of clinical symptoms. PNP may have a fatality rate of up to 90% due to respiratory failure or sepsis. 

Histopathology will reveal a combination of features depending on the type of lesion biopsied: suprabasal acantholysis for blisters and erosions and an interface vacuolar dermatitis for erythema multiforme-like lesions, and lichenoid interface dermatitis with dyskeratotic keratinocytes for the lichenoid lesions. Nguyen et al found CD8+ cytotoxic T lymphocytes in the epidermis, and CD4+ lymphocytes within the dermis, along with CD56+ natural killer cells, and CD68+ monocytes/macrophages. 

Etiopathogenetic theories on the association of PNP with underlying malignancies have included: 1) lymphoproliferative malignancies express desmoplakins, which induce autoimmunity and lead to dysregulation of tumor cytokine production (increased levels of IL-6 promote B-cell differentiation and production of anti-plakin autoantibodies), which bind the target antigens exposed by epitope-spreading phenomenon (antigen diversification) leading to cell membrane damage. 2) anti-tumor response involving CD4+ and CD8+ T cells is mediated both by antibody-dependent cellular cytotoxicity (ADCC) and direct cytotoxic reactions by PNP IgG resulting in target cell damage; 3) Nguyen et al propose that damage to basal layer keratinocytes by natural killer and ADCC effector T cells exposes hidden self-antigen which results in autoantibody production to desmosomal and hemidesmosomal proteins like intracellular plakins, as well as Dsg. Hence, a combination of humoral and CMI mechanisms may be responsible for clinical disease.

PNP sera contains IgG which react with characteristic set of antigens by immunoprecipitation, most of which are cytoplasmic components of the plakin family of adhesion proteins (demoplakin I and II, bullous pemphigoid BP230, envoplakin, periplakin, and HE1/plectin) and an as yet unidentified 170 kDa antigen. Dsg-1 and 3 have been reported to play a role in early stage PNP in about 5-20% of patients and most PNP serum reacts consistently with the N-terminal, central and C-terminal domains of both envoplakin and periplakin. IB studies reveal reactivity with 210 kDa envoplakin, 190
kDa periplakin and BP180 NC16a recombinant protein. Passive transfer studies have proven pathogenic circulating autoantibodies cause suprabasilar acantholysis in neonatal mice, while immunoadsorption with recombinant Dsg 1 and Dsg3 from PNP sera will prevent blister formation.

DIF will reveal a combination of IgG and C3 deposition at the BMZ (pemphigoid-like pattern), intercellular staining in the epidermis (pemphigus-like) and homogeneous staining within keratinocyte cytoplasm (apoptosis-like pattern) (Fig. 4). Taintor et al found intercellular keratinocyte cell staining and weak BMZ staining with IgA in a patient with CLL. IIF will show keratinocyte cell surface and BMZ IgG deposition in substrates like human skin and monkey esophagus. Likewise, using transitional epithelium such as rat bladder, PNP sera will reveal IgG autoantibodies to anti-keratinocyte cell surface. Nguyen et al found that titers of intercellular antibodies, not anti-BMZ antibodies, correlate well with the clinical course of PNP. IIF studies of PNP serum on desmocollin-transfected COS-7 cells demonstrated IgG and IgA antibodies to Dsc-3 and Dsc-2. IB analysis of PNP sera using human epidermal extracts and recombinant proteins (N-terminal globular domains, entire central rod domains and C-terminal globular domains) of both envoplakin and periplakin will detect IgG autoantibodies and other studies have detected plectin autoantibodies in some PNP patients.

Immunoprecipitation reveals IgG autoantibodies reacting against antigens from the intercellular proteins of the plakin gene family: HD-1/plectin (>400 kDa), desmoplakin-1 (250 kDa), BPAG-1 (230 kDa), envoplakin, desmoplakin II (210-kDa double band), periplakin (190kDa) and a still unidentified keratinocyte polypeptide antigen with apparent MW of 170kDa. In addition, Nguyen et al found unique immunoprecipitated keratinocyte proteins with apparent MWs of 40/42, 66, 70, 80, 95, and 150 kDa. Samples from other PNP patients also reacted with 130 kDa and 170 kDa proteins, as well as 240 kDa proteins. The 130 kDa antigen found by IP in their PNP patients was an unknown antigen bearing the same MW as Dsg-3.

Seishima analysed PNP sera using ELISA to Dsg-1, Dsg-3 and BP180, which resulted in positivity for IgG to desmoglein 3 but not to Dsg-1 and BP180. Using ELISA to Dsg-1 and 3, they found only antibodies to Dsg-3 relating to the presence of exclusively mucosal lesions, but additional Dsg-1 positivity upon appearance of skin lesions, suggesting a pathogenesis similar to classic pemphigus, with the clinical phenotype relating to anti-Dsg antibody levels. Ohyama et al used the same Dsg ELISA in 21 PNP patients but found no clear association between

Fig. 4
Intercellular keratinocyte surface staining with broad granular deposition of IgG in a case of paraneoplastic pemphigus. (fluorescence microscopy, original magnification x 400). (Used with permission from Antoinette F. Hood, MD and Steven D. Billings, MD)
clinical phenotype and anti-Dsg autoantibody profile in PNP suggesting other pathological mechanisms (such as cell-mediated cytotoxicity) in PNP. ELISA testing was done in 14 cases of childhood PNP which showed that 10/14 had autoantibodies to Dsg-3 and 13/14 patients had autoantibodies to Dsg1, which has been supported by other authors. PNP serum using IgA ELISA to Dsg-1 and Dsg 3 have been done with only 1 study detecting anti-Dsg-1 IgA antibodies.

Taintor et al used expression vectors containing coding sequence to Dsc-1,2 and 3, transfecting COS-7 cells by a lipofection method, and found IgA antibodies staining cell surface of COS-7 cells transfected with Dsc-2 in a PNP patient with CLL and predominantly IgA autoantibody profile.

CONCLUSION

Significant progress has been made in identifying target antigens, subclass and pathogenic autoantibodies in the pemphigus group of autoimmune blistering diseases. Desmogleins are a major antigen in this group, however desmocollins are playing a larger role in more pemphigus variants. These discoveries further elucidate the pathogenesis of these diseases, and are also leading to the description of new variants, such as IgG/IgA pemphigus. The desmoglein ELISAs are particularly useful in monitoring autoantibody levels that reflect disease activity, severity and clinical phenotype. In general, ELISA techniques rapidly and efficiently confirm the diagnosis in classic pemphigus, however unless all the relevant antigens are identified, its use will remain limited to sera containing autoantibodies staining intercellular cell surfaces on DIF or IIF. Thus, a combination of traditional IF techniques (used as an initial screening tool) with ELISA (to Dsg-1 and 3) is the rational approach to the diagnosis of patients suspected of having pemphigus and its variants.

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