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Review Article

Newer Diagnostic Methods in Detection and Classification of Vesiculo-Bullous Lesions

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Abstract: The practice of pathology is currently undergoing significant change, in large part due to advances in the analysis of DNA, RNA, and proteins in tissues. These advances have permitted improved biologic insights into many developmental, inflammatory, metabolic, infectious and neoplastic diseases. Moreover, molecular analysis has also led to improvements in accuracy of disease diagnosis and classification. It is likely that, in the future, these methods will increasingly enter into the day-to-day diagnosis and management of patients. The pathologist will continue to play a fundamental role in diagnosis and will likely be in a pivotal position to guide the implementation and interpretation of these tests as they move from the research laboratory into diagnostic pathology.
Keywords: Vesiculo-Bullous Lesions, Immunoflorescence, Molecular Methods, Pemphigus, Pemphigoid

INTRODUCTION

The diagnosis of vesiculo-bullous lesions and many other diseases is fundamentally based on the microscopic study of cells and tissues. This diagnostic method remains the standard, by which all other diagnostic tests are measured. Nevertheless, the era of the pathologist relying entirely on the examination of tissue sections stained by histochemical methods is gradually being replaced by a time when advanced immunologic and molecular techniques (i.e. analysis of DNA, RNA or protein structure and function) augment the process by which complicated diseases are classified. In the next several years, it can be expected that molecular criteria will increasingly be utilized for diagnosis and prognosis. Many of these molecular advances had their start in basic science research laboratories, after validation, technological improvements and automation; they have made their way into applied molecular pathology research and increasingly into the day-to-day practice of pathology. Today, the extent to which immunopathological and molecular pathologic techniques are used varies greatly, but it is conceivable that in the next decade many of today's most technically advanced methods of molecular analysis will become standard practice. This review describes in detail about the classification of various vesiculo-bullous lesions of oral cavity, mucous membranes and skin along with their identification by newer diagnostic methods.

HISTORY AND CLINICAL FINDINGS

Bullous autoimmune dermatoses exhibit a chronic progression, in which the principal clinical symptoms are blisters and painful secondary erosions on the skin and mucous membranes that lack a tendency to heal. The most frequent adult bullous skin disease, the bullous pemphigoid (BP), demonstrates taut blisters on the torso and extremities. A prodromal phase with pruritus and papulovesicular or urticarial plaques can precede the occurrence of blisters. In diseases of the

pemphigus group, loose fragile blisters are often no longer detectable and erosions with a crusty surface are frequently prominent. Table 1 shows the clinical characteristics of different bullous autoimmune dermatoses. The most important differential diagnoses of bullous autoimmune dermatoses include: a) infectious diseases in connection with bacterial and viral infections; b) immunological causes, such as bullous drug induced skin rashes or erythema exudativum multiforme (EEM); and c) the group of hereditary blister- forming dermatoses. Table 2 summarizes these differential diagnoses.

Table 1: Clinical characteristic of Bullous autoimmune skin diseases

Disease	Clinical Characteristics
Pemphigus Vulgaris (PV) / Foliaceus (PF)	Loose blisters/erosions on mucous membranes (PV) and skin (PF, PV); PF predominantly in seborrheic skin areas
Paraneoplastic Pemphigus (PNP)	Haemorrhagic stomatitis, polymorph exanthema; lichenoid efflorescences palmoplantar; frequent associations: haematologic malignoma, thymoma, Castleman tumour
IgA-Pemphigus	Fragile, annular, rim-accentuated blisters or pustules, or crusty erosions on the skin; rarely affection of mucous membranes
Bullous Pemphigoid (BP)	Urticarial, pruriginous plaques with taut blisters or erosions; intertriginous areas on the flexor side of the extremities; 10–30% involvement of the mucous membranes
Pemphigoid Gestationis (PG)	Erythematous papules and plaques, eczema, pruritus; often 2nd or 3rd trimester
Mucous Membrane Pemphigoid (MMP, formerly Erosions and ulcerations with atrophy of the mucous cicatricial pemphigoid, CP)	membranes (beware conjunctivitis, symblephara), involvement of skin in ;25% of cases, capillitium, head, upper trunk
Linear IgA Bullous Dermatosis (LABD/CBDC)	Most frequent form in childhood; polymorph, often centrifugally grouped, pearl string-like arranged taut blisters, often mucous membrane involvement; associations: colitis ulcerosa; primary sclerosing cholangitis
Epidermolysis Bullosa Acquisita (EBA)	Mechanobullous, inflammatory and atrophic variant, frequent involvement of mucous membranes; scarring and formation of milia
Dermatitis Herpetiformis (DH)	Accentuated excoriated papule vesicles at extensor side or small blisters; pruriginous papulae, erythematous plaques, intense pruritus; coeliac disease does not have to be clinically manifest

Table 2: Differential diagnoses of Vesiculo-Bullous diseases

Disease groups	Disease	Clinical/diagnostic Characteristics
Hereditary Bullous	Epidermolysis bullosa simplex group, basal membrane bullous epidermolysis group, dystrophic bullous epidermolysis group	Occurrence at birth or early childhood; clinical findings according to genetic defect; DIF and IIF negative
Infectious Diseases	Impetigo contagiosa	Microbiology, other signs of inflammation; IF and serology negative
	Staphylococcal scalded-skin syndrome	Most widely described epidermolysis, histology; IF and serology negative
	Bullous erysipelas	Clinical and serological inflammation parameters; IF and serology negative
	Herpes simplex	Clinical and serological inflammation parameters; IF and serology negative
	Varicella zoster virus	Clinical picture, general symptoms; IF and serology negative
Immunological Diseases	Bullous systemic lupus erythematosus (SLE)	IF and serology positive (anti-collagen VII-Ak), ANA positive; other SLE criteria positive
	Erosive lichen ruber planus	IF: sub epidermal cytoid bodies, IIF negative; cutaneous involvement, hepatopathy
	Erythema exsudativum multiforme (EEM)	History; IF and serology with majus form sometimes positive, ICS pattern (anti-desmoplakin-Ak)

	Bullous drug-induced exanthema (SJS, TEN)	EEM-like picture or laminar epidermolysis, histology; IF and serology negative
	Sub corneal pustulosis (Sneddon-Wilkinson)	Leukocytosis, serologic signs of inflammation, DIF and serology negative
Other Diseases	Porphyria cutanea tarda	Porphyrins in serum and urine, IF and serology negative, light-exposed areas
	Bullosis diabeticorum	Glucose in serum/urine, IF and serology negative
	Traumatic/toxic blister formation	History; DIF and serology negative

CLASSIFICATION OF BULLOUS AUTOIMMUNE DERMATOSES

Classification of the blister-forming autoimmune oro-mucocutaneous lesions is based on the localization of the blister formation [1]. The diseases are categorized into three groups: those that exhibit an intra-epidermal loss of adhesion (acantholysis); those with skin-splitting in the area of the basement membrane zone; and dermatoses with damaged sublaminal adherence. An overview of this classification is given in Table 3. In diagnosing bullous autoimmune oral and skin diseases, histological examination of diseased skin allows differentiation between intra- and sub epidermal blister formation. Thus, histology plays a major role in differentiating intra-epidermal (desmosomal) from sub epidermal loss of adhesion. However, definitive classification is not possible from histological findings alone.

Table 3: Immunofluorescence in bullous autoimmune diseases

Disease	Direct Immunofluorescence	Indirect Immunofluorescence	Target antigens
Pemphigus Vulgaris (PV), Pemphigus Foliaceus(PF)	Intercellular IgG and C3	Intercellular IgG (monkey oesophagus)	Dsg3 (PV), Dsg1 (PF, PV), Dsg4 (assoc. with anti-Dsg1) (seldom plakins)
Paraneoplastic Pemphigus	Intercellular IgG and C3; linear IgG and C3 at the BMZ	Intercellular IgG (rat bladder)	Dsg1/Dsg3, plakins (desmoplakin) envoplakin, periplakin), BP230, 170 kDa-antigen
IgA-Pemphigus	Intercellular IgA and C3	Intercellular IgA (monkey oesophagus) in 50%	Desmocollin1, Dsg1/Dsg3
Bullous Pemphigoid	Linear IgG (IgA) and C3 at the BMZ	IgG epidermal (SSS)	BP180, BP230
Pemphigoid Gestationis	Linear IgG < C3 at the BMZ	IgG epidermal (SSS)	BP180 (BP230)
Mucous membrane Pemphigoid	Linear IgG and/or IgA and/or C3 at BMZ	IgG or IgA epidermal± dermal (SSS)	BP180 (BP230), laminin-5,α6b4-integrin
Linear IgA-Bullous dermatosis	Linear IgA (and C3) at theBMZ	IgA epidermal, where applicable dermal(SSS)	LAD-1, BP180, BP230
Epidermolysis Bullosa Acquisita	Linear IgG and C3 at the BMZ;linear IgA and C3 at the BMZ (IgA- type EBA)	IgG (or IgA) and C3 dermal(SSS)	Collagen VII
Dermatitis Herpetiformis	Granular IgA deposits at dermal papillae	IgA against endomysium of smooth muscle cells (monkey oesophagus)	Epidermal transglutaminase

DIAGNOSTIC WORKUP

Bullous autoimmune disorders, including some life-threatening diseases, manifest in the skin and mucous membranes and are clinically characterized by the appearance of blisters and secondary erosions. Bullous autoimmune dermatoses have a common pathogenic mechanism involving binding of auto antibodies to specific adhesion molecules in epidermal desmosomes, and in some cases in the area of the dermo-epidermal basement membrane zone. The

binding of circulating auto antibodies and the induction of an inflammatory reaction in the area of target structures lead to loss of adhesion with subsequent intra- or sub epidermal blister formation [2]. The clinical appearance is heterogeneous and secondary eruptions such as erosions, encrustation, impetiginisation, and scarred secondary erosions can dominate the clinical picture.

DIRECT AND INDIRECT IMMUNOFLUORESCENCE

Direct Immunofluorescence

The diagnosis of bullous autoimmune mucosal diseases involves immunohistological and immunoserological tests [3]. Using direct immunofluorescence (DIF) of perilesional skin tissue-bound autoantibodies, mostly classified as IgG, but also including IgA (linear IgA dermatosis, IgA pemphigus, IgA-type Epidermolysis bullosa acqusita), as well as the complement factor C3, can be verified. The localization of tissue-bound autoantibodies leads to characteristic fluorescent patterns, which allow allocation to specific entities. Intercellular reticulate (desmosomal) deposits of IgG (or IgA in IgA pemphigus) and complement factors (frequently C3) are obvious in all pemphigus diseases. Linear deposits of IgG and C3 in the area of the dermo-epidermal basement membrane zone can be found in the pemphigoid group and Epidermolysis bullosa acquisita.

Linear deposits of IgA at the basal membrane are characteristic of linear IgA dermatosis and are also found in IgA-type Epidermolysis bullosa acqusita. The DIF of Dermatitis herpetiformis Duhring shows granular IgA deposits in the dermal papillae and occasionally in the basement membrane area.

Indirect Immunofluorescence

Indirect immunofluorescence (IIF) detects circulating serum autoantibodies in patients. In this test, monkey esophagus is used as a substrate for pemphigus diseases. In certain cases, plakin-rich tissue, such as guinea pig oesophagus or rat bladder epithelia, is used. An additional substrate for IIF is sodium chloride split skin. This test is used to further differentiate pemphigoid, linear IgA dermatosis, and EBA. Pemphigoid disease and EBA demonstrate immunohistologically linear deposits of IgG and C3 in the area of the dermo-epidermal basement membrane zone. In addition, linear IgA deposits in the area of the basement membrane zone are characteristic of linear IgA dermatosis, but can also be found in the IgA variant of EBA. For the sodium chloride split-skin test, human skin is incubated in 1 M NaCl solution, so that an artificial split is induced in the area of the lamina lucida of the basement membrane zone.

This splitting separates pemphigoid autoantigens from those of EBA. Sera of pemphigoid patients typically show IgG binding to target antigens in the area of the blister roof (epidermal part), whereas sera of patients with EBA show IgG reactivity at the base of the blister (dermal part). IgA autoantibodies can be verified in 60–70% of patients with linear IgA dermatosis, with reactivity to autoantigens in the epidermal area of the NaCl-split skin. A specific characteristic of sera from patients with mucous membrane pemphigoid is that laminin-5 (epiligrin) - reactive sera show reactivity to the epidermal and dermal parts of the NaCl-split skin, where the IgG reactivity shows a typical pattern at the blister base.

IMMUNOSEROLOGICAL TESTS WITH RECOMBINANT AUTOANTIGENS OR AUTOANTIGEN EXTRACTS

ELISA and immunoblot tests with recombinant and purified native autoantigens are an important addition to the diagnosis of bullous autoimmune dermatoses. The identification of autoantigens of most autoimmune blistering skin diseases has allowed their use in immunoserological procedures in the last few years [4-8]. These antigens are either prepared by recombination or from epidermal extracts or cultured keratinocytes. Commercial test systems are currently only available for the autoantigens Dsg1/Dsg3 (pemphigus group) for the NC16A region of BP (bullous pemphigoid) and for tissue transglutaminase (dermatitis Herpetiformis).

A type VII-collagen has been described for EBA, but is not commercially available [9]. Therefore, dermal protein extracts for immunoblot analysis are used for proof of autoantibodies in EBA. Furthermore, specialised laboratories, including the Departments of Dermatology in Marburg, Freiburg, and Lu beck, carry out additional serological tests in the form of ELISAs, immunoblots, and immunoprecipitation with recombinant and native varieties of the autoantigens Dsg1/Dsg3, BP180/BP230 and laminin-5 [10-12].

The current immunoserologic course of tests commonly used is based on recent developments in the diagnostic field of bullous autoimmune mucosal diseases. ELISA tests are at present the most important method for diagnosis. A correlation between disease activity and antibody titres was verified, especially for pemphigus and pemphigoid diseases [13]. The frequency of serological controls during the disease course mainly depends on the clinical picture. In phases of active disease, monitoring of autoantibodies is recommended every 4 weeks. With stable skin conditions, quarterly check-ups seem reasonable. Fundamentally there are three goals.

In the first instance, ELISA tests, as well as semi-quantitative immunoblots and immunoprecipitation, allow a correlation between the clinical picture and the extent of the antibody titre. Secondly, these examination methods allow characterization of the autoantibody subclasses. In the acute stages of pemphigus disease there is already evidence that besides IgG4, desmoglein- reactive autoantibodies of the class IgG1 can be detected, as well as sporadic detection of IgE [7]. Thirdly, characterization of the epitope specificity of autoantibodies is possible [14, 15]. Therefore, immunoserology not only confirms diagnosis, but also provides a better understanding of the immunopathogenesis of these diseases.

MOLECULAR METHODS IN DIAGNOSTIC PATHOLOGY

Proteins are the effector molecules of all cells, and their amino acid sequence and tertiary structures, to a large degree, are determined by the nucleotide sequences contained in the nuclear DNA of the cell. Since the late 1950s and early 1960s, molecular biologists have learned to characterize, isolate, and manipulate the molecular components of cells and organisms. These components include DNA, the repository of genetic information; RNA, a close relative of DNA whose functions range from serving as a temporary working copy of DNA to actual structural and enzymatic functions as well as a functional and structural part of the translational apparatus; and proteins, the major structural and enzymatic type of molecule in cells [16]. The use of DNA- and RNA-based tests continues to grow for applications as varied as inherited disease, infectious disease, cancer, identity testing, human leukocyte antigen typing, and pharmacogenetics. Progress is driven in part by the huge growth in knowledge about the molecular basis of disease coupled with advancements in technologic capabilities. In addition to requirements for clinical utility, every molecular test also may have limitations that must be carefully considered before clinical implementation. Analytic and clinical performance characteristics as well as test limitations are established and documented through the process of test validation [17].

EXPRESSION CLONING

One of the most basic techniques of molecular biology to study protein function is expression cloning. In this technique, DNA coding for a protein of interest is cloned (using PCR and/or restriction enzymes) into a plasmid (known as an expression vector). A vector has 3 distinctive features: an origin of replication, a multiple cloning site (MCS), and a selective marker (usually antibiotic resistance). The origin of replication will have promoter regions upstream from the replication/transcription start site [18].

This plasmid can be inserted into either bacterial or animal cells. Introducing DNA into bacterial cells can be done by transformation (via uptake of naked DNA), conjugation (via cell-cell contact) or by transduction (via viral vector). Introducing DNA into eukaryotic cells, such as animal cells, by physical or chemical means is called transfection. Several different transfection techniques are available, such as calcium phosphate transfection, electroporation, microinjection and liposome transfection. DNA can also be introduced into eukaryotic cells using viruses or bacteria as carriers, the latter is sometimes called bactofection and in particular uses Agrobacterium tumefaciens. The plasmid may be integrated into the genome, resulting in a stable transfection, or may remain independent of the genome, called transient transfection [18].

In either case, DNA coding for a protein of interest is now inside a cell, and the protein can now be expressed. A variety of systems, such as inducible promoters and specific cell-signaling factors, are available to help express the protein of interest at high levels. Large quantities of a protein can then be extracted from the bacterial or eukaryotic cell. The protein can be tested for enzymatic activity under a variety of situations, the protein may be crystallized so its tertiary structure can be studied, or, in the pharmaceutical industry, the activity of new drugs against the protein can be studied [18].

POLYMERASE CHAIN REACTION (PCR)

The polymerase chain reaction is an extremely versatile technique for copying DNA. In brief, PCR allows a single DNA sequence to be copied (millions of times), or altered in predetermined ways. For example, PCR can be used to introduce restriction enzyme sites, or to mutate (change) particular bases of DNA, the latter is a method referred to as "Quick change". PCR can also be used to determine whether a particular DNA fragment is found in a cDNA library. PCR has many variations, like reverse transcription PCR (RT-PCR) for amplification of RNA, and, more recently, real-time PCR (QPCR) which allow for quantitative measurement of DNA or RNA molecules [19].

GEL ELECTROPHORESIS

Gel electrophoresis is one of the principal tools of molecular biology. The basic principle is that DNA, RNA, and proteins can all be separated by means of an electric field. In agarose gel electrophoresis, DNA and RNA can be separated on the basis of size by running the DNA through an agarose gel. Proteins can be separated on the basis of size by using an SDS-PAGE gel, or on the basis of size and their electric charge by using what is known as a 2D gel electrophoresis [20].

MACROMOLECULE BLOTTING AND PROBING

The terms northern, western and eastern blotting are derived from what initially was a molecular biology joke that played on the term Southern blotting, after the technique described by Edwin Southern for the hybridization of blotted DNA. Patricia Thomas, developer of the RNA blot which then became known as the northern blot actually didn't use the term. Further combinations of these techniques produced such terms as southwesterns (protein-DNA hybridizations), northwesterns (to detect protein-RNA interactions) and farwesterns (protein-protein interactions), all of which are presently found in the literature.

SOUTHERN BLOTTING

Named after its inventor, biologist Edwin Southern, the Southern blot is a method for probing for the presence of a specific DNA sequence within a DNA sample. DNA samples before or after restriction enzyme digestion are separated by gel electrophoresis and then transferred to a membrane by blotting via capillary action. The membrane is then exposed to a labeled DNA probe that has a complement base sequence to the sequence on the DNA of interest. Most original protocols used radioactive labels; however non-radioactive alternatives are now available. Southern blotting is less commonly used in laboratory science due to the capacity of other techniques, such as PCR, to detect specific DNA sequences from DNA samples. These blots are still used for some applications, however, such as measuring transgene copy number in transgenic mice, or in the engineering of gene knockoutembryonic stem cell lines [21].

NORTHERN BLOTTING

The northern blot is used to study the expression patterns of a specific type of RNA molecule as relative comparison among a set of different samples of RNA. It is essentially a combination of denaturing RNA gel electrophoresis, and a blot. In this process RNA is separated based on size and is then transferred to a membrane that is then probed with a labeled complement of a sequence of interest. The results may be visualized through a variety of ways depending on the label used; however, most result in the revelation of bands representing the sizes of the RNA detected in sample. The intensity of these bands is related to the amount of the target RNA in the samples analyzed. The procedure is commonly used to study when and how much gene expression is occurring by measuring how much of that RNA is present in different samples. It is one of the most basic tools for determining at what time, and under what conditions, certain genes are expressed in living tissues [22].

WESTERN BLOTTING

Antibodies to most proteins can be created by injecting small amounts of the protein into an animal such as a mouse, rabbit, sheep, or donkey (polyclonal antibodies) or produced in cell culture (monoclonal antibodies). These antibodies can be used for a variety of analytical and preparative techniques. In western blotting, proteins are first separated by size, in a thin gel sandwiched between two glass plates in a technique known as SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The proteins in the gel are then transferred to a PVDF, nitrocellulose, nylon or other support membrane. This membrane can then be probed with solutions of antibodies. Antibodies that specifically bind to the protein of interest can then be visualized by a variety of techniques, including colored products, chemiluminescence, or autoradiography. Often, the antibodies are labeled with enzymes. When a chemiluminescent substrate is exposed to the enzyme it allows detection. Using western blotting techniques allows not only detection but also quantitative analysis. Analogous methods to western blotting can be used to directly stain specific proteins in live cells or tissue sections. However, these immunostaining methods, such as FISH, are used more often in cell biology research [23].

EASTERN BLOTTING

Eastern blotting technique is to detect post-translational modification of proteins. Proteins blotted on to the PVDF or nitrocellulose membrane are probed for modifications using specific substrates [24].

ARRAYS

A DNA array is a collection of spots attached to a solid support such as a microscope slide where each spot contains one or more single-stranded DNA oligonucleotide fragment. Arrays make it possible to put down large quantities of very small (100 micrometre diameter) spots on a single slide. Each spot has a DNA fragment molecule that is complementary to a single DNA sequence (similar to Southern blotting). A variation of this technique allows the gene expression of an organism at a particular stage in development to be qualified (expression profiling). In this technique the RNA in a tissue is isolated and converted to labeled cDNA [25].

This cDNA is then hybridized to the fragments on the array and visualization of the hybridization can be done. Since multiple arrays can be made with exactly the same position of fragments they are particularly useful for comparing the gene expression of two different tissues, such as a healthy and cancerous tissue. Also, one can measure what genes are expressed and how that expression changes with time or with other factors. For instance, the common baker's yeast, Saccharomyces cerevisiae, contains about 7000 genes; with a microarray, one can measure qualitatively how

each gene is expressed, and how that expression changes, for example, with a change in temperature. There are many different ways to fabricate microarrays; the most common are silicon chips, microscope slides with spots of ~ 100 micrometre diameter, custom arrays, and arrays with larger spots on porous membranes (macroarrays). There can be anywhere from 100 spots to more than 10,000 on a given array [25].

Arrays can also be made with molecules other than DNA. For example, an antibody array can be used to determine what proteins or bacteria are present in a blood sample.

ALLELE-SPECIFIC OLIGONUCLEOTIDE

Allele-specific oligonucleotide (ASO) is a technique that allows detection of single base mutations without the need for PCR or gel electrophoresis. Short (20-25 nucleotides in length), labeled probes are exposed to the non-fragmented target DNA. Hybridization occurs with high specificity due to the short length of the probes and even a single base change will hinder hybridization. The target DNA is then washed and the labeled probes that didn't hybridize are removed. The target DNA is then analyzed for the presence of the probe via radioactivity or fluorescence. In this experiment, as in most molecular biology techniques, a control must be used to ensure successful experimentation. The Illumina Methylation Assay is an example of a method that takes advantage of the ASO technique to measure one base pair differences in sequence [26].

CONCLUSION

This review has described many important molecular tools that are currently been used or will be used in diagnostic pathology. However the pathologist will continue to play a central role in diagnosis and it is conceivable the armamentarium of diagnostic test will induce many of these advances. Thus these changes will likely improve the understanding of diseases.

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