Molecular Diagnosis of Autoimmune Blistering Diseases

Daisuke Tsuruta, Teruki Dainichi, Takahiro Hamada, Norito Ishii, and Takashi Hashimoto

Abstract

Autoimmune bullous diseases are the best-characterized autoimmune skin diseases. Molecular diagnosis of these diseases has become possible due to the identification of their target autoantigens over the past three decades. In this review, we summarize methodology for categorizing autoimmune bullous diseases by means of combinations of direct and indirect immunofluorescence techniques using normal human skin sections, rat bladder sections and COS7 cells transfected with desmocollins 1–3 encoded vectors, enzyme-linked immunosorbent assays and immunoblotting with normal human epidermal extracts, dermal extracts, purified proteins from cell cultures and recombinant proteins.

Key words: Molecular diagnosis, Autoimmune bullous disease, Immunofluorescence, COS7 cell, Immunoblot, ELISA

1. Introduction

In healthy individuals, the immune system can accurately distinguish “self” from “non-self” and attacks only the latter (1). Autoimmune disease are caused by dysregulation of this system (1). The ligation of surface receptors on lymphocytes or the binding of antibodies to “self” epitopes can cause inflammation and tissue damage, resulting in autoimmune disease (1). Thus far, more than 80 types of autoimmune diseases have been reported. Although the causes mostly remain obscure, some diseases are known to be triggered by bacteria or viruses with epitopic similarities to body constituents (“molecular mimicry”) (2). Autoimmune diseases can be divided into two major types, systemic and tissue-specific (3). The topic of the present review is the latter, occurring in the skin.

Autoimmune skin diseases include autoimmune bullous diseases, such as pemphigus and pemphigoid, cutaneous connective tissue diseases, vasculitis, psoriasis, vitiligo, autoimmune urticaria,
and alopecia areata. In this review, we focus on autoimmune bullous diseases. Combinations of various diagnostic tools are used for the diagnosis.


The major cell–cell adhesion moieties in keratinocytes are the desmosomes (4), the major components of which are grouped three protein families: cadherins, plakins, and armadillo proteins (Fig. 1) (4). Desmosomal cadherins are divided into two transmembrane protein families: desmogleins 1–4 and desmocollins 1–3 (4). Their cytoplasmic tails bind to armadillo family members, plakoglobin, plakophilins 1–3, and p0071 (4). Desmoplakin, a plakin family protein, tethers these molecules to keratin intermediate filaments in the cytoplasm (5).

Isoform-specific expression of desmogleins and desmocollins is observed in the epithelium and epidermis (4). Simple epithelia express only desmoglein 2 and desmocollin 2 (4). In contrast, the epidermis shows high expression of desmogleins 1 and 3, and desmocollins 1 and 3, but low expression of desmoglein 2 and desmocollin 2 (4). Desmoglein 4 is concentrated in the granular and cornified layers as well as hair follicles (4). Desmogleins are the major targets in pemphigus, the prototype of autoimmune bullous disease (6).
The major structures for cell–extracellular matrix adhesion in keratinocytes are hemidesmosomes (Fig. 2) (7). Major hemidesmosomal transmembrane proteins are BP180/collagen XVII, integrin α6 subunit, integrin β4 subunit, and CD151 tetraspanin (8). Both BP180 and α6β4 integrin interact with laminin-332 in the basement membrane zone (9). In the cytoplasm, α6β4 integrin associates with keratin intermediate filaments (10). The cytoplasmic tail of β4 integrin has a unique long stretch of 1,000 amino acids (7). Through this cytoplasmic tail, α6β4 integrin binds to BP180, BP230, and plectin (7). The latter two proteins belong to the plakin family and mediate the indirect connection of α6β4 integrin not only to keratin intermediate filaments but also microtubules and actin microfilaments (10). Hemidesmosomal components are targets of autoantibodies in subepidermal autoimmune bullous diseases.

Pemphigus is an autoimmune bullous disease whose autoantigens are the desmogleins, main desmosomal constituents (6). The pemphigus disease variants and the associated autoantigens are summarized in Table 1. Histopathologically, it is characterized by acantholysis.
and intraepidermal blister formation (11). Pemphigus is divided into two main types: pemphigus vulgaris (PV) and pemphigus foliaceus (PF) (12). Pemphigus vegetans is a rare variant of PV, and pemphigus erythematosus resembles PF (13). In addition, further very rare entities of the pemphigus group are represented by IgA pemphigus and tumor-related paraneoplastic pemphigus (13, 14).

The expression of desmogleins 1 and 3 is different in the skin and the oral mucosa (Fig. 3). Desmoglein compensation theory can explain the difference of clinical findings between PV reactive with desmoglein 3 and PF reactive with desmoglein 1. In the skin, desmoglein 1 is strongly expressed throughout the epidermis, being stronger in the superficial epidermis (15). The expression of desmoglein 3 is primarily observed in the basal and suprabasal epidermis. In contrast, in the oral mucosa, desmoglein 1 and 3 are found throughout the entire epithelium, although the expression of desmoglein 1 is much weaker than desmoglein 3. First, why mucosal-dominant lesions are found in PV, while skin-dominant lesions are found in PF? This is essentially due to the fact that in the oral mucosa, anti-desmoglein 3 antibodies in PV disrupt epithelial cell–cell adhesions, which are not compensated by small amount of desmoglein 1, whereas anti-desmoglein 1 antibodies cannot disrupt desmoglein 3-rich epithelial cell. Second, in the skin, anti-desmoglein 3 antibodies cannot disrupt cell–cell adhesion because desmoglein 1 compensates the loss of desmoglein 3-mediated adhesion. In contrast, anti-desmoglein 1 antibodies cause disruption in the upper epidermis, where no desmoglein 3 is present (Fig. 2).
In PV, most patients suffer from refractory erosions or ulcers on the oral mucosae including the lips and tongue (12, 16). Some PV patients also show flaccid bullae and erosions on the skin (12). PV patients may also have erosions on other mucosae including larynx, pharynx, esophagus, conjunctiva, and vagina (17–19). Pemphigus vegetans is a variant of PV (13).

The diagnosis of PV and pemphigus vegetans is made by combination method of direct immunofluorescence showing the deposition of IgG and/or C3 at the keratinocyte cell surfaces (20) and enzyme-linked immunosorbent assays for IgG antibodies to desmoglein 3 and desmoglein 1. This is required for the correct diagnosis of all pemphigus group diseases (21). In mucosal-dominant type PV, anti-desmoglein 3 but not anti-desmoglein 1 antibodies are present (21). In contrast, in mucocutaneous type PV, antibodies for both desmoglein 1 and desmoglein 3 are present (21).

In PF, most patients suffer from superficial erosions or bullae and erythema, preferentially on the seborrheic regions (6). Oral mucosal lesions are not present (6). Pemphigus erythematosus is a variant of PF. The butterfly shadow is characteristic of pemphigus erythematosus (13). The diagnosis of PF and pemphigus erythematosus is made via the same methodology as PV, described above.
Clinically, pemphigus herpetiformis is characterized by small vesicles arranged in an annular fashion on the pruritic erythemas, resembling clinically dermatitis herpetiformis Duhring (22). The diagnosis of pemphigus herpetiformis is exclusively on the basis of its characteristic clinical features associated with histopathological intraepidermal eosinophilic pustules with minimal acantholysis (23). IgG autoantibodies to desmocollins may contribute to pemphigus herpetiformis, although they are not always found (22, 24). The detection of autoantibodies to desmocollins is done by indirect immunofluorescence using desmocollin cDNA-transfected COS7 cells (24).

IgA pemphigus is clinically defined by generalized multiple flaccid pustules or vesicles (25). The hallmark finding of IgA pemphigus is deposition of IgA on keratinocyte cell surfaces by direct immunofluorescence (25). IgA pemphigus is subdivided into subcorneal pustular dermatosis type and intraepidermal neutrophilic IgA dermatosis type (25). The autoantigen of the former is desmocollin 1, but that of the latter is unidentified yet (25). IgA autoantibodies to desmocollin 1 were first detected by indirect immunofluorescence using desmocollin 1 cDNA-transfected COS7 cells (26).

Paraneoplastic pemphigus is characterized by pseudomembranous conjunctivitis and refractory stomatitis (27). The skin symptoms are variable, including erythema, flaccid bullae, tense bullae, erosion, erythema multiforme-like lesions, and/or lichen planus-like lesions (28). Paraneoplastic pemphigus is associated with the presence of internal benign or malignant tumors, including Castleman’s disease, malignant lymphomas and other solid cancers (14). If treatment is ineffective, cases with bronchiolitis obliterans are mostly fatal (29). Diagnosis of paraneoplastic pemphigus is now made by the positive IgG reaction by indirect immunofluorescence using rat bladder sections and a double-positive reaction to the 210 kDa enoplakin and 190 kDa periplakin by immunoblotting using normal epidermal extracts (30, 31). In addition, anti-desmoplakin and anti-plectin antibodies are sometimes also found by immunoblotting (14).
The autoimmune bullous diseases which target the basement membrane zone and their autoantigens are presented in Table 2. Bullous pemphigoid is the most common among all autoimmune bullous diseases (32).

**11. Bullous Pemphigoid**

Bullous pemphigoid is the most common autoimmune bullous disease seen in the elderly, and is characterized by itchy erythema and tense bullae, caused by IgG autoantibodies to the hemidesmosomal proteins, BP180 and BP230 (33). Bullous pemphigoid patients occasionally develop mucosal lesions (33). Bullous pemphigoid is believed to have higher association of internal malignancy (34). As a pathomechanism, autoantibodies to BP180 cause migration of neutrophils and eosinophils and activation of proteases, resulting in proteolysis of the basement membrane zone (35, 36). The diagnosis of bullous pemphigoid is made by direct and indirect immunofluorescence, immunoblotting and ELISA (37, 38). Direct immunofluorescence using patient skin shows deposition of C3 and/or IgG to the basement membrane zone (37). Indirect immunofluorescence using normal human skin sections detects circulating IgG antibodies to the basement membrane zone (37). Additionally, by indirect immunofluorescence using sections of 1 M NaCl-split normal human skin, patient sera react with the epidermal side of the split (37, 38). By immunoblotting, IgG in the patient sera reacts with BP180 and/or BP230 (33). ELISA with either the recombinant NC16a domain of BP180 and mixture of C- and N-terminal domains of BP230 protein shows a sensitivity of about 85% (39, 40). However, when the both tests were performed, sensitivity raises to 96% (40).

**12. Mucous Membrane Pemphigoid**

Patients with mucous membrane pemphigoid occasionally develop skin lesions similar to bullous pemphigoid, which tend to heal with scars (41). However, predominant clinical manifestations are erythemas, bullae, and erosions on the oral, nasal, and ocular mucosae (42). Blindness caused by adhesive conjunctivitis is the most severe complication for mucous membrane pemphigoid (43). The two major target autoantigens are the C-terminus of BP180 and...
Classification, antibody classes, and autoantigens for autoimmune bullous diseases which target keratinocyte–extracellular matrix adhesion

<table>
<thead>
<tr>
<th>Disease</th>
<th>Ab class</th>
<th>Autoantigen</th>
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<tbody>
<tr>
<td>Bullous pemphigoid</td>
<td>IgG</td>
<td>BP180, BP230</td>
</tr>
<tr>
<td>Herpes gestationis</td>
<td>IgG</td>
<td>BP180</td>
</tr>
<tr>
<td>Mucous membrane pemphigoid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-BP180 type</td>
<td>IgG, IgA</td>
<td>BP180</td>
</tr>
<tr>
<td>anti-laminin-332 type</td>
<td>IgG</td>
<td>Laminin-332</td>
</tr>
<tr>
<td>ocular type</td>
<td>IgG</td>
<td>Integrin β4 subunit</td>
</tr>
<tr>
<td>Linear IgA bullous dermatosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lamina lucida type</td>
<td>IgA</td>
<td>97/120 kDa LAD-1</td>
</tr>
<tr>
<td>sub-lamina densa type</td>
<td>IgA</td>
<td>Unidentified (type VII collagen)</td>
</tr>
<tr>
<td>Epidermolysis bullosa acquisita</td>
<td>IgG</td>
<td>Type VII collagen</td>
</tr>
<tr>
<td>Bullous SLE</td>
<td>IgG</td>
<td>Type VII collagen</td>
</tr>
<tr>
<td>Anti-laminin γ1 pemphigoid</td>
<td>IgG</td>
<td>Laminin γ1 subunit</td>
</tr>
<tr>
<td>Dermatitis herpetiformis Duhring</td>
<td>IgA</td>
<td>Transglutaminase 3</td>
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laminin-332 (44, 45). Diagnosis is made by direct and indirect immunofluorescence and immunoblotting. The findings of direct and indirect immunofluorescence using normal human skin sections and 1 M salt split skin sections for anti-BP180-type mucous membrane pemphigoid are the same as for bullous pemphigoid, except that IgA is also frequently detected (46, 47). The IgG and IgA antibodies react with BP180 in epidermal extracts and recombinant protein of BP180 C-terminus domain by immunoblotting (48). IgG antibodies in the sera of anti-laminin-332 mucous membrane pemphigoid react with dermal side of split skin and with laminin-332 by immunoblotting using human laminin-332 purified from normal human keratinocyte culture media (49). In addition, autoantibodies to α6β4 integrin are reported to be associated with ocular-type mucous membrane pemphigoid (50).

### 13. Anti-Laminin γ1 (p200) Pemphigoid

Anti-laminin γ1 pemphigoid is characterized by tense bullae and erosions and is frequently associated with psoriasis (51). Histopathologically, it is characterized by subepidermal blisters with neutrophilic infiltrations (51). Although direct immunofluorescence and indirect immunofluorescence using normal human skin sections give the same results as bullous pemphigoid,
patient IgG reacts with the dermal side of split skin (50). Immunoblotting using dermal extracts shows IgG reactivity with a 200 kDa protein (50), which was identified as laminin γ1 (52).

### 14. Herpes Gestationis

Herpes (pemphigoid) gestationis occurs during pregnancy and early postpartum, or in patients with hydatidiform moles or choriocarcinoma (53). Clinically, it is characterized by severe pruritus and tense bullae on the urticarial infiltrative erythema (54). The main autoantigen is the NC16a domain of BP180, as in bullous pemphigoid (54–56). Most patients enter remission after pregnancy, but a few cases show a prolonged clinical course (54). Although the reason why herpes gestationis occurs only in pregnancy is not known, some studies have suggested the role of HLA related immunogenetics (57, 58).

### 15. Dermatitis Herpetiformis Duhring

Dermatitis herpetiformis clinically shows tense vesicles on the periphery of annular infiltrative exudative erythema, which exhibit symmetrical distribution on the knees, elbows, and buttocks (59). In Caucasian, but not Japanese, patients dermatitis herpetiformis Duhring is associated with celiac disease (60). Direct immunofluorescence shows granular deposits of IgA and C3 in the papillary dermis (61). In addition, recently, the target autoantigen has been identified as epidermal transglutaminase (transglutaminase 3) (62, 63).

### 16. Linear IgA Bullous Dermatosis

Clinically, linear IgA bullous dermatosis develops pruritic small vesicles in the periphery of annular infiltrative erythemas, similar to dermatitis herpetiformis Duhring (64). The linear deposition of IgA at the basement membrane zone seen in direct and indirect immunofluorescence is a hallmark for the diagnosis of the disease and the origin of its name (64). The target autoantigen is 97/120 kDa LAD-1, a shedding product of BP180, excised probably by a protease of ADAM family (65). IgA autoantibodies to LAD-1 are detected by immunoblotting using concentrated HaCaT cell culture media (64). In addition, an ELISA system was developed for the detection of IgA autoantibodies against BP180 in linear IgA bullous dermatosis (66).
Epidermolysis bullosa acquisita is divided into inflammatory and non-inflammatory types; the former shows bullous pemphigoid-like skin lesion, and the latter shows non-erythematous blisters leaving scarring and milia (67). The target autoantigen is type VII collagen, a major component of the anchoring fibrils (67). Findings in indirect immunofluorescence using sections of normal human skin and salt-split skin are the same as those in anti-laminin-γ1 pemphigoid and anti-laminin-332 mucous membrane pemphigoid (67). The detection of autoantibodies to type VII collagen by immunoblotting using normal dermal extracts as substrates is helpful for diagnosis (67).

Direct immunofluorescence for IgG, IgA, and C3 is performed in order to distinguish between pemphigus group diseases, various types of pemphigoid, and dermatitis herpetiformis Duhring (6, 33, 68). Deposition of IgG and IgA to the keratinocyte cell surfaces is indicative of the diagnosis of various types of pemphigus (6), and IgA pemphigus (25), respectively. Deposition of IgG and IgA to the basement membrane zone is characteristic for diseases of the pemphigoid group (33), and linear IgA bullous dermatosis (69), respectively. Dermatitis herpetiformis Duhring shows granular or fibrillar deposition of IgA and/or C3 in dermal papillae (68).

By indirect immunofluorescence using normal human skin sections, IgG from pemphigus patients reacts with the keratinocyte cell surfaces, while IgG from pemphigoid patients reacts with the basement membrane zone (6, 33). In mucous membrane pemphigoid, IgG and IgA to the basement membrane zone are frequently negative because of their low titers (70). IgA from linear IgA bullous dermatosis patients reacts with the basement membrane zone, although false-negative reactions are also occasionally seen, due to the low titer of the autoantibodies (71).

Indirect immunofluorescence using salt-split normal human skin sections is used for differential diagnosis of pemphigoid group diseases. 1 M NaCl treatment results in a split at the level of the lamina lucida. Sera from patients with bullous pemphigoid, anti-BP180-type mucous membrane pemphigoid and linear IgA bullous dermatosis react to the epidermal side of the split, while sera from patients with anti-laminin γ1 pemphigoid, epidermolysis bullosa acquisita, and anti-laminin-332 mucous membrane pemphigoid react with dermal side (33, 52, 64, 67, 72). Complement immunofluorescence is used for diagnosis of herpes
gestationis (55). Moreover, indirect immunofluorescence using rat bladder sections detects anti-plakin antibodies in paraneoplastic pemphigus, and indirect immunofluorescence using COS7 cells transfected with cDNAs of desmocollins 1–3 is used to detect IgA anti-desmocollin 1 antibodies in subcorneal pustular dermatosis type IgA pemphigus and IgG antibodies to desmocollins 1–3 in pemphigus herpetiformis, pemphigus vegetans, or paraneoplastic pemphigus (26, 31).

18.2. Enzyme-Linked Immunosorbent Assays

The enzyme-linked immunosorbent assay is used to diagnose and to monitor the clinical course for pemphigus group diseases and bullous pemphigoid (21, 39, 40). The recombinant proteins are prepared by baculovirus expression for desmoglein 1 and desmoglein 3 or by E. coli expression for NC16a domain of BP180 and N- and C-terminal domains of BP230 (21, 39, 40). A limitation of current enzyme-linked immunosorbent assays is that the results are not always correlated with disease severity. This is thought to be mostly due to the presence of nonpathogenic antibodies (73). Therefore, future development of enzyme-linked immunosorbent assays specific for the pathogenic epitopes is required. Enzyme-linked immunosorbent assay for envoplakin is now commercially available, too (74). At the experimental stage, ELISA systems for detecting desmocollins, periplakin, type VII collagen, LAD-1, laminin γ1, and A2ML1 are already available, but are not yet released for routine clinical diagnostic use (42, 66, 75, 76).

18.3. Immunoblot Analyses

Immunoblotting is performed as follows: normal human epidermal or dermal extracts, or keratinocyte cell lysates are electrophoretically separated and then transferred to nitrocellulose or PVDF membranes. Patient sera are then reacted with these membranes. In addition, recombinant proteins for various antigens, purified laminin-332 and concentrated culture medium of HaCaT cells are also used for subepidermal autoimmune bullous diseases. The substrates used in immunoblotting studies for each disease are summarized in Table 3.

19. Conclusions

An algorithm for the differential diagnosis for each disease is shown in Fig. 4. Using this methodology, we can logically diagnose all autoimmune bullous diseases. However, rapid progress in molecular techniques suggests that this algorithm will need sequential modification and updating. In particular, in the near future, diagnostic enzyme-linked immunosorbent assays for the entire list of the aforementioned autoantigens should be introduced for clinical use.
Table 3
The substrates used in immunoblotting studies for autoimmune bullous diseases

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human epidermal extract</td>
<td>Pemphigus, BP, herpes gestationis</td>
</tr>
<tr>
<td>Human dermal extract</td>
<td>EBA, anti-laminin γ1 pemphigoid</td>
</tr>
<tr>
<td>HaCaT cell culture medium</td>
<td>LABD</td>
</tr>
<tr>
<td>Purified laminin-332</td>
<td>Laminin-332 type MMP</td>
</tr>
<tr>
<td>Recombinant proteins</td>
<td></td>
</tr>
<tr>
<td>BP180 NC16a domain</td>
<td>BP, herpes gestationis</td>
</tr>
<tr>
<td>BP180 C-terminus</td>
<td>BP180 type MMP</td>
</tr>
<tr>
<td>BP230</td>
<td>BP</td>
</tr>
<tr>
<td>Type VII collagen</td>
<td>EBA</td>
</tr>
<tr>
<td>Envoplakin, periplakin</td>
<td>PNP</td>
</tr>
</tbody>
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BP bullous pemphigoid, EBA epidermolysis bullosa acquisita, LABD linear IgA bullous dermatosis, MMP mucous membrane pemphigoid, PNP paraneoplastic pemphigus

Fig. 4 Algorithm for the diagnosis for all autoimmune bullous diseases. BMZ basement membrane zone, CIF complement immunofluorescence, CS cell surface, C-ter carboxy terminus, der dermal, Dsc desmocollin, Dsg1 desmoglein 1, Dsg3 desmoglein 3, EBA epidermolysis bullosa acquisita, epi epidermal, IB immunoblot, IEN intraepithelial neutrophil IgA dermatisis, IIF indirect immunofluorescence, LAD linear IgA bullous dermatosis, lam lamnin, MMP mucous membrane pemphigoid, m-PV mucosal-dominant type pemphigus vulgaris, mc-PV mucocutaneous type pemphigus vulgaris, PE pemphigus erythematosus, pem pemphigus, PF pemphigus foliaceus, PH pemphigus herpetiformis, PNP paraneoplastic pemphigus, Pveg pemphigus vegetans, rec recombinant, SPD subcorneal pustular dermatosis, VII col type VII collagen.
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References


Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain. J Invest Dermatol 106:141–146


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