Immunopathology and molecular diagnosis of autoimmune bullous diseases

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Abstract

Autoimmune bullous diseases are associated with autoimmunity against structural components maintaining cell–cell and cell–matrix adhesion in the skin and mucous membranes. Pemphigus diseases are characterized by autoantibodies against the intercellular junctions and intraepithelial blisters. In pemphigoid diseases and epidermolysis bullosa acquisita, sub-epidermal blistering is associated with autoantibodies targeting proteins of the hemidesmosomal anchoring complex. The autoantigens in autoimmune blistering diseases have been extensively characterized over the past three decades. In general, the pathogenicity of autoantibodies, already suggested by clinical observations, has been conclusively demonstrated experimentally. Detection of tissue-bound and circulating serum autoantibodies and characterization of their molecular specificity is mandatory for the diagnosis of autoimmune blistering diseases. For this purpose, various immunofluorescence methods as well as immunoassays, including immunoblotting, enzyme-linked immunosorbent assay and immunoprecipitation have been developed. This review article describes the immunopathological features of autoimmune bullous diseases and the immunological and molecular tests used for their diagnosis and monitoring.

Keywords: autoimmunity ● immunofluorescence ● immunoblotting ● ELISA

Introduction

Autoimmune blistering diseases are acquired chronic diseases associated with an immune response directed to structural proteins that maintain cell–cell and cell–matrix adhesion in the skin and mucous

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Table 1 Immunopathological features of autoimmune bullous diseases (reviewed in [2])

Disease	Direct immunfluorescence	Indirect immunofluorescence	Autoantigens
Pemphigus diseases			
Pemphigus vulgaris	Intercellular IgG and C3	Intercellular IgG (monkey esophagus)	Dsg[‡] 3, Dsg 1
Pemphigus foliaceus	Intercellular IgG and C3	Intercellular IgG (monkey esophagus)	Dsg 1
Paraneoplastic pemhigus	IgG and C3 intercellularly and at the dermal–epidermal junction	Intercellular IgG (monkey esophagus and rat bladder[§])	Dsg 3, Dsg 1, plakines
IgA pemphigus	Intercellular IgA and C3	Intercellular IgA (monkey esophagus)	Dsc[¶] 1, Dsg 3
Pemphigoid diseases			
Bullous pemphigoid	Linear C3 and IgG at the dermal–epidermal junction	Epidermal IgG (SSS[††])	BP180, BP230
Pemphigoid gestationis	Linear C3 at the dermal--epidermal junction	Epidermal complement fixing IgG (SSS)	BP180, BP230
Mucous membrane pemphigoid	Linear IgG, IgA and C3 at the dermal–epidermal junction	Epidermal or dermal IgG, IgA (SSS)	BP180, Laminin 5, $\alpha_6\beta_4$ integrin
Linear IgA disease	Linear IgA (and C3) at the dermal–epidermal junction	Epidermal IgA (SSS) Dermal IgA (SSS)	LAD-1 Type VII collagen
Epidermolysis bullosa acquisita	Linear IgG, IgA and C3 at the dermal–epidermal junction	Dermal IgG (SSS)	Type VII collagen
Dermatitis herpetiformis	Granular IgA deposits in the dermal papillae	Anti-endomysium IgA (monkey esophagus)	Transglutaminase

[‡]Dsg, desmoglein.

[§]Rat bladder as a sensitive substrate for detection of circulating autoantibodies in paraneoplastic pemphigus.

[¶]Dsc, desmocollin.

[††]SSS, skin incubated with 1 M NaCl, as a substrate for detection of circulating autoantibodies in subepidermal blistering diseases.

membranes. Based on clinical, histopathological, and immunopathological criteria, autoimmune bullous diseases are classified into four major groups: pemphigus diseases and pemphigoid diseases, epidermolysis bullosa acquisita, and dermatitis herpetiformis Duhring (Table 1). The first group of diseases includes life-threatening blistering diseases characterized by intraepidermal blister formation due to the loss of adhesion of keratinocytes and is associated with autoantibodies to the intercellular junctions of keratinocytes. The remainder of these diseases are characterized by sub-epidermal blisters caused by the loss of attachment of basal keratinocytes to the underlying basement membrane and are associated with deposition of immunoreactants at the dermal–epidermal junction. Target antigens of autoantibodies have been identified for the majority of autoimmune blistering diseases (Table 1, Fig. 1). In general, the pathogenicity of autoantibodies, already suggested by clinical observations, has been conclusively demonstrated experimentally.

The diagnosis of an autoimmune blistering disease is suggested by the clinical and histopathological features. For routine histological examination, a
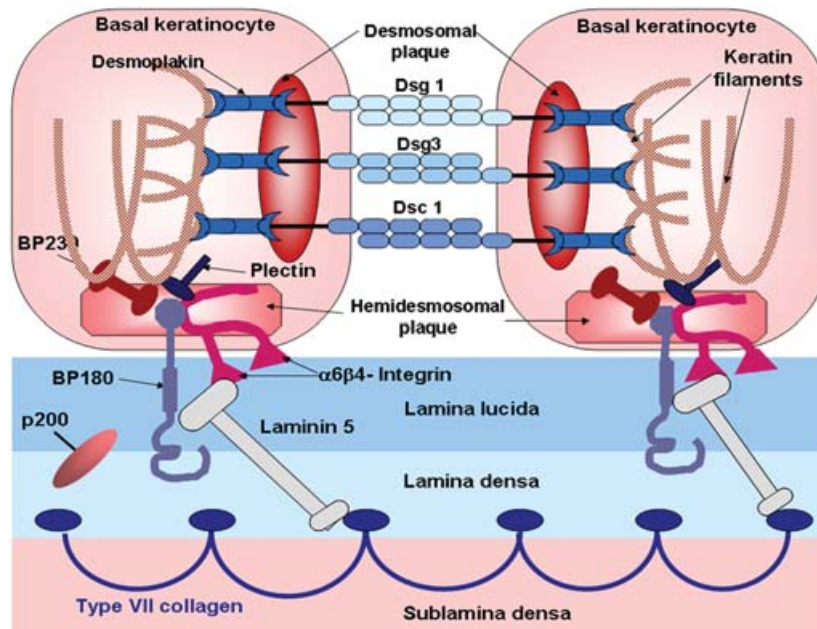
Fig. 1 Schematic diagram of the desmosome and the dermal–epidermal junction. Here are represented only structural proteins that function as autoantigens in autoimmune bullous skin diseases. Neighbouring keratinocytes are associated via the extracellular portions of desmosomal cadherins. As examples, homophilic interactions between desmoglein 1, desmoglein 3 and desmocollin 1 are depicted. Their intracellular portions bind to desmosomal plaque proteins that mediate the interaction of desmosomes with keratin filaments. Keratin filaments also bind to bullous pemphigoid antigen 230 (BP230) and plectin, the main intracellular constituents of the hemidesmosomes. BP230 and plectin function as ligands for transmembrane hemidesomosomal proteins, type XVII collagen (BP180) and $\alpha_6\beta_4$ integrin. These may connect the hemidesmosomes to laminin 5, which in addition to type IV collagen, is a major component of the lamina densa. Laminin 5 is a known ligand for type VII collagen, the major constituent of the anchoring fibrils, which connect lamina densa to the collagen bundles of the upper dermis.

fresh vesicle/blister (less than 24 hrs old) is biopsied, preferably in its entirety, placed in formaldehyde, and processed for hematoxylin & eosin staining [1, 2]. However, the diagnosis of an autoimmune blistering disease requires detection of tissue bound and circulating autoantibodies in the skin and/or mucous membranes. Deposition of immunoreactants in tissues and circulating serum autoantibodies are detected by direct and indirect immunofluorescence microscopy, respectively. For the direct immunofluorescence microscopy, the biopsy is taken from perilesional (more than 1 cm from the lesion) or uninvolved skin. The biopsy must be snap frozen immediately and stored at temperatures below –70˚C or placed in a special transport medium suitable for later immunofluorescence testing [2]. Failure to collect or preserve samples adequately may result in rapid degradation and loss of immunoreactants, leading to false-nega-

tive results. Circulating serum autoantibodies can be detected by indirect immunofluorescence microscopy performed on frozen sections of normal tissues, including human skin, monkey esophagus, and rodent or monkey bladder. When this technique is performed on salt-split skin that has been previously incubated in 1 M NaCl, the sensitivity is increased and further information about the antibody-binding site can be obtained [2].

Autoantibodies directed to different autoantigens that co-localize microscopically cannot be differentiated based on patterns obtained by indirect immunofluorescence microscopy. However, identification of target antigen(s) is necessary for the diagnosis. Characterization of molecular specificity of circulating autoantibodies is performed using immunoassays, including immunofluorescence, immunoblotting, enzyme-linked immunosorbent assay (ELISA),

and immunoprecipitation. Both native antigens from skin extracts or cultured keratinocytes and different recombinant forms of these proteins serve as substrate for these immunoassays [2].

Pemphigus diseases

Pemphigus (from the Greek pemphix meaning bubble or blister) designates a group of life-threatening autoimmune blistering diseases characterized by intraepithelial blister formation [3–5]. The molecular basis for blister formation is a loss of adhesion between epidermal cells (acantholysis) caused by circulating autoantibodies directed against intercellular adhesion structures of keratinocytes [6–9]. Several forms of pemphigus have been described depending on the level of the intraepidermal split formation, including two major sub-types, pemphigus vulgaris and pemphigus foliaceus. Splitting is suprabasal in pemphigus vulgaris and its rare vegetating form, pemphigus vegetans. Blistering is more superficial in pemphigus foliaceus and related sub-types [6–9].

The direct immunofluorescence microscopy of patients' perilesional skin reveals intercellular deposition of immunoreactants. Circulating serum autoantibodies can be detected by indirect immunofluorescence microscopy using human skin or other senzitive substrates, most commonly monkey esophagus (Fig. 2). Autoantibodies in patients with pemphigus vulgaris and foliaceus target different keratinocyte proteins, including desmogleins 1 [10], 3 [11, 12] and 4 [13], annexins [14] and acetylcholine receptors [15]. Cloning of desmoglein 1 and 3 cDNA enabled the development of a sensitive and specific ELISA and extensive characterization of the autoreactivity against these antigens in pemphigus patients [16]. Serum levels of autoantibodies detected by ELISA strongly correlate with disease activity in pemphigus patients [16, 17]. The development of commercially available diagnostic tools for the remainder of the autoantigens in pemphigus should facilitate their further characterization in pemphigus patients with regard to reactivity of specific autoantibodies and patterns in disease and remission.

The pathogenicity of autoantibodies from patients with pemphigus was demonstrated for the first time by Anhalt and colleagues who showed that IgG purified from pemphigus vulgaris patients causes blisters when
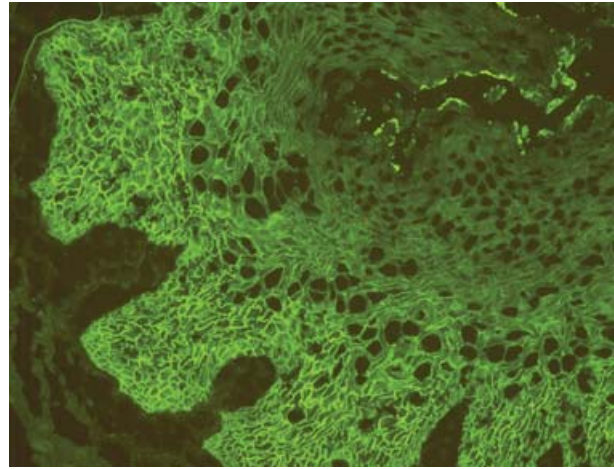

Fig. 2 Serological tests used for the diagnosis of pemphigus. Circulating autoantibodies show an intercellular binding pattern by indirect immunofluorescence microscopy on monkey esophagus (magnification, 3200).

administered to neonatal mice by intraperitoneal or sub-cutaneous injection [18]. Subsequently, the pathogenicity of autoantibodies from patients with sporadic or endemic pemphigus foliaceus (fogo selvagem) was demonstrated using a similar approach [19, 20].

Pemphigus vulgaris

Pemphigus vulgaris is clinically characterized by flaccid blisters/erosions of the mucous membranes and the skin. In the majority of patients, the oral mucosa is primarily affected but other mucous membranes may be involved as well. Once the disease progresses, skin lesions may develop at any site of the tegument, including the intertriginous areas, the trunk and the scalp [4].

Histopathological examination reveals acantholysis with a sparse inflammatory infiltrate. Split formation occurs in the suprabasal layer leaving a single layer of basal keratinocytes attached to the dermal–epidermal basement membrane ('row of tombstones'). Direct immunofluorescence microscopy of patients' perilesional skin reveals intercellular deposits of IgG and C3 [21]. Circulating autoantibodies in serum of pemphigus vulgaris patients binding to the intercellular junctions of keratinocytes in human skin or other substrates, like monkey esophagus, may be detected by indirect immunofluorescence
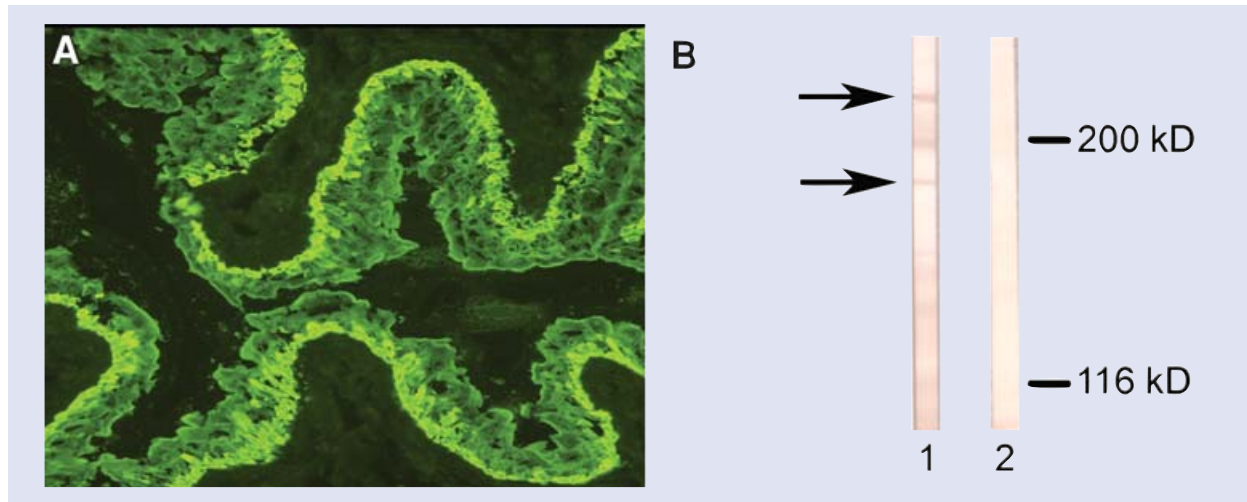
Fig. 3 Serological tests used for the diagnosis of paraneoplatic pemphigus. **(A)** Serum autoantibodies from a patient with paraneoplastic pemphigus bind to rat bladder urothelium, as revealed by indirect immunofluorescence microscopy (magnification, ×200). **(B)** An extract of cultured keratinocytes was fractionated by 7.5% SDS-PAGE, transferred to nitrocellulose, and incubated with paraneoplastic pemphigus serum (lane 1) and with normal human serum (lane 2). Paraneoplastic pemphigus serum reacts with envoplakin (210 kD; upper arrow) and periplakin (190 kD; lower arrow), while control serum shows no specific reactivity. Molecular weight markers are depicted on the right side of the panel.

microscopy [22]. At molecular level, the specificity of IgG autoantibodies from pemphigus vulgaris patients seems to be heterogenous. Autoantibodies in virtually all patients with pemphigus vulgaris were shown to react with desmoglein 3 and a subgroup also with desmoglein 1 [12, 23–25]. Though less characterized compared with desmogleins, more recently other autoantigens, including α9-acetylcholine receptor and pemphaxin, were demonstrated in pemphigus vulgaris patients [14, 15].

Pemphigus foliaceus

Pemphigus foliaceus is less severe than pemphigus vulgaris. The onset is usually insidious with scattered, scaly lesions involving the seborrhoeic areas: scalp, face, chest and upper back. Localized disease slowly extends. Blistering may not be obvious because the cleavage is superficial and the small, flaccid blisters rupture. Scales separate leaving crusted erosions surrounded by erythema, sometimes with small vesicles along the borders. Erosions are both painful and offensive. Oral lesions are uncommon. By histopathology, the split occurs sub-corneally and is associated with

an inflammatory infiltrate consisting mainly of neutrophil granulocytes. IgG autoantibodies in serum of pemphigus foliaceus patients recognize the 160-kD desmosomal glycoprotein subsequently identified as desmoglein 1 [10, 26–28].

Pemphigus variants

Pemphigus herpetiformis

Pemphigus herpetiformis is a form of pemphigus resembling dermatitis herpetiformis in its early-phase [29]. It is characterised by widespread clusters of pruritic papules and vesicles, which develop on an erythematous background. Histopathology shows sub-corneal pustules, eosinophilic spongiosis or features of dermatitis herpetiformis without acantholysis, but immunofluorescence studies reveal staining of keratinocytes surfaces for IgG. Patients circulating IgG autoantibodies recognize desmoglein 1, and, in a subgroup of patients, desmoglein 3 [30]. The condition usually evolves into classical pemphigus foliaceus, and rarely into pemphigus vulgaris.

Pemphigus erythematosus

Pemphigus erythematosus (Senear-Usher syndrome) is a form of pemphigus foliceus characterized by sharply demarcated erythematous plaques with scaling. The lesions are primarily localized on the face and the upper trunk [6, 31]. In addition, in some of these patients, deposits of immunoreactants along the skin basement membrane zone and serum antinuclear antibodies may be found suggesting an association with lupus erythematosus [2].

Drug-induced pemphigus

Although there are sporadic case reports of pemphigus in association with the use of certain medication, the association with sulfhydril drugs, including D-penicillamin and captopril, is the most significant [32]. Clinically, superficial pemphigus (including erythematosus) is more common than pemphigus vulgaris in sulfhydril-drugs treated patients. Though the autoantigen(s) of the drug-induced pemphigus have not yet been thoroughly characterized, autoantibodies against desmogleins were found in a subgroup of patients [33].

Paraneoplastic pemphigus

Paraneoplastic pemphigus is usually characterized by painful lesions of the mucosal surfaces and erythema multiforme-like lesions of palms and soles [34–37]. This relatively rare disease is mainly associated with B cell lymphoma and other haematological disorders, but it may also occur in patients with benign tumours, like thymoma or Castleman's tumour [34–39]. Direct immunofluorescence microscopy shows, in addition to keratinocyte cell surface IgG, deposition of C3, both in the intercellular space and at the dermal–epidermal junction. Unlike the classic types of pemphigus, in which indirect immunofluorescence is positive only on stratified squamous epithelial substrates, paraneoplastic pemphigus sera also often show staining of other types of desmosome-containing tissues, including bladder, heart and liver (Fig. 3A). Autoantibodies from patients with paraneoplastic pemphigus immunoprecipitate a characteristic set of antigens, including desmoplakin I, bullous pemphigoid antigen 1 (BP230), periplakin, envoplakin, and a not yet identified protein of 170 kD [34]. Although immunoprecipitation is still the gold standard for the demonstration of specific autoantibodies, immunoblotting is a valuable aid for diagnosis. Paraneoplastic pemphigus sera recognize periplakin and envoplakin from epidermal or keratinocytes extracts or their recombinant forms [2, 40–42] (Fig. 3B).

IgA pemphigus

IgA pemphigus is clinically characterized by pustules with a tendency to confluence forming annular and circinate patterns [43, 44]. The immunopathological hallmark of the disease is IgA deposition on the keratinocytes cell surfaces [44]. Circulating IgA autoantibodies in sub-corneal pustular dermatosis type of IgA pemphigus were shown to target desmocollins [45]. In contrast, the IgA autoimmune response in patients with intraepidermal neutrophilic dermatosis sub-type appears to be more heterogenous. While desmogleins 1 and 3 represent minor antigens [46–48], immunoelectron microscopy studies suggest that IgA autoantibodies in these patients recognize a non-desmosomal transmembranous protein [49].

Pemphigoid diseases

Pemphigoid diseases are characterized by separation at the dermal–epidermal junction, and are associated with an autoimmune response to structural components of the anchoring filaments. The histopathological examination shows sub-epidermal blisters and a rich inflammatory infiltrate. The direct immunofluorescence microscopy reveals linear deposition of immunoreactants at the dermal–epidermal junctions. Based on the clinical and immunopathological features and on the targeted autoantigens, there are various forms of pemphigoid diseases (Table 1).

Bullous pemphigoid

Bullous pemphigoid was first described as an entity separate from pemphigus and dermatitis herpetiformis by Lever in 1953, based on its clinical and histopathological features [3]. Bullous pemphigoid mainly affects elderly and is the most common autoimmune blistering disease in the Western
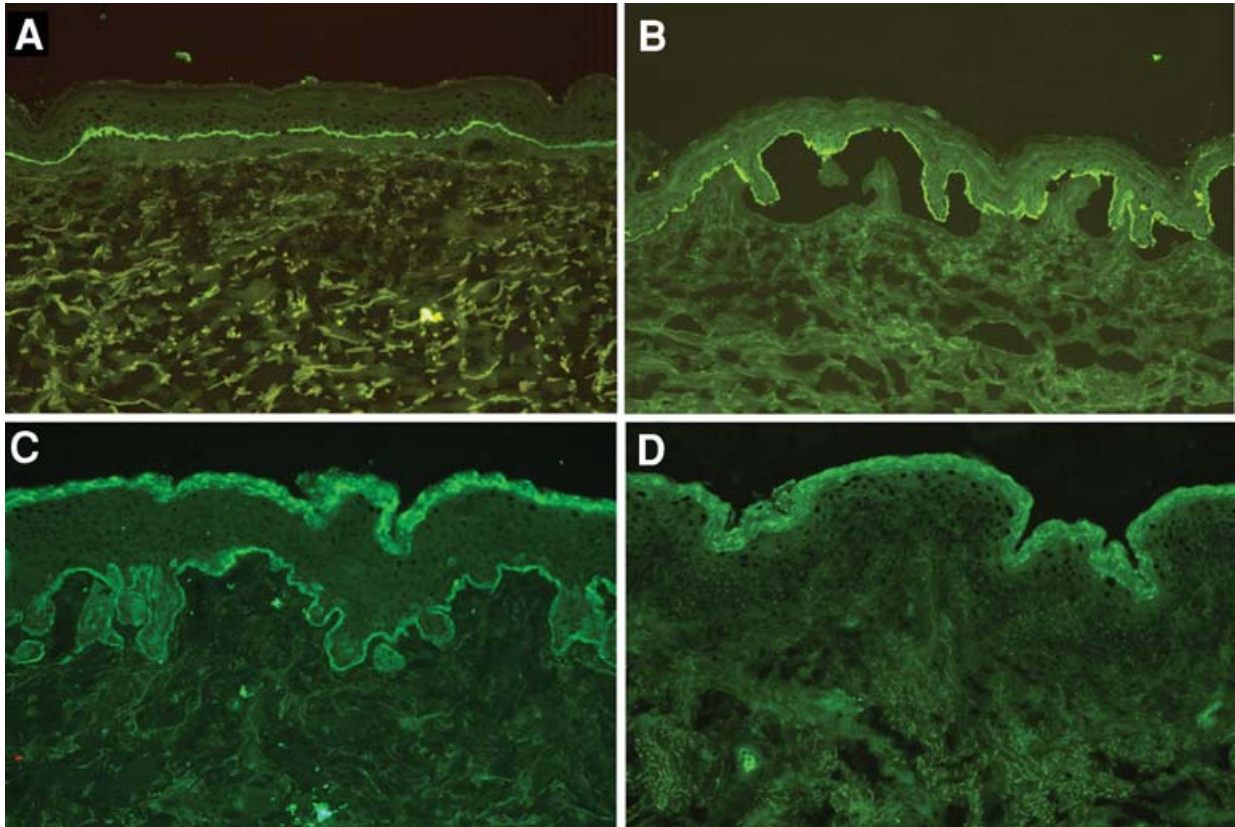
Fig. 4 IF microscopy analysis in bullous pemphigoid and pemphigoid gestationis. **(A)** Direct immunofluorescence microscopy of perilesional skin reveals deposition of complement C3 at the dermal–epidermal junction of a patient with bullous pemphigoid (magnification, ×100). **(B)** Serum bullous pemphigoid IgG autoantibodies bind to the epidermal side of 1M NaCl-split skin, by indirect immunofluorescence microscopy (magnification, ×100). Cryosections of normal human skin were incubated with **(C)** pemphigoid gestationis serum or **(D)** normal human serum, and, subsequently, with fresh serum from a healthy control, used as a source of complement. Staining with an FITC-labeled antibody to human C3 reveals linear complement deposits at the dermal–epidermal junction in the case of the section incubated with the pemphigoid gestationis serum, but not with normal human serum (magnification, ×200).

Europe and North America. Clinically, patients with bullous pemphigoid present large tense blisters arising on normal or erythematous skin, preferentially on the lower abdomen, the inner aspects of thighs and the intertriginous areas.

By the use of immunoprecipitation and immunoblotting techniques, two hemidesmosomal proteins, BP230 and BP180, were identified as being the targets of circulating autoantibodies in bullous pemphigoid patients [50–53]. Whereas BP230 is located intracellularly, BP180, also referred to as type XVII collagen, is a transmembrane protein consisting of an intracellular globular domain, a transmembrane segment, and an extracellular domain that contains

15 collagenous sub-domains separated by non-collagenous domains. The 16th non-collagenous A domain adjacent to the cell membrane of basal keratinocytes has been identified as the immunodominant region of BP180, targeted by approximately 80–90% of bullous pemphigoid sera [54–56].

Several lines of evidence support a pathogenic role of autoantibodies to BP180: (1) rabbit antibodies generated against murine and hamster forms of BP180 induce sub-epidermal blistering when passively transferred into neonatal mice or hamsters, respectively [57, 58]; (2) autoantibodies to BP180 from patients with bullous pemphigoid recruit leucocytes to the dermal–epidermal junction and induce
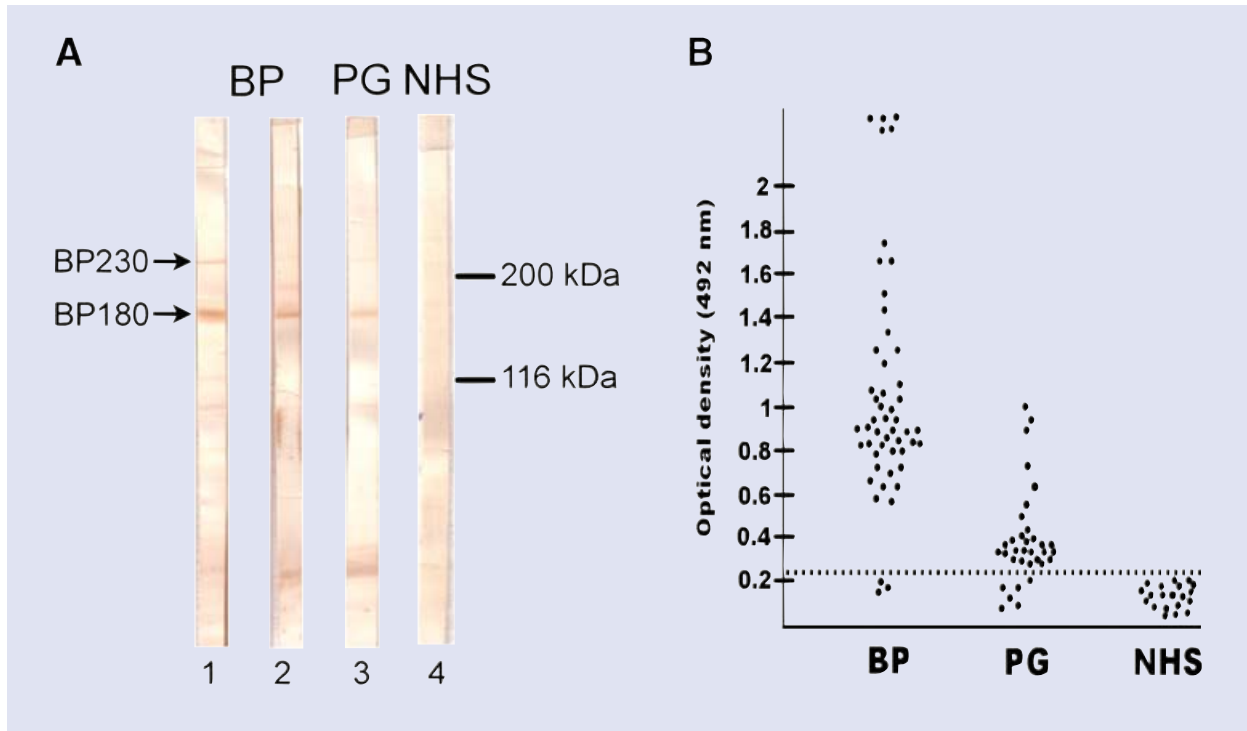
Fig. 5 Serological tests used for the diagnosis of bullous pemphigoid and pemhigoid gestationis. **(A)** An extract of cultured keratinocytes was fractionated by 7.5% SDS-PAGE, transferred to nitrocellulose, and incubated with serum from patients with bullous pemphigoid (BP; lanes 1–2), pemphigoid gestationis (PG; lane 3) and a healthy control (NHS; lane 4). The bullous pemphigoid serum depicted in lane 1 reacts with both bullous pemphigoid antigen 230 (BP230, 230 kD, upper arrow) and bullous pemphigoid antigen 180 (BP180, 180kD, lower arrow), while the bullous pemphigoid serum shown in lane 2 and the pemphigoid gestationis serum shown in lane 3 react with BP180 only. Control serum shows no specific reactivity (lane 4). Molecular weight markers are depicted on the right side of the panel. **(B)** The ELISA using a recombinant form of the 16th non-collagenous A domain of the bullous pemphigoid antigen 180 is a specific and sensitive tool for the diagnosis of bullous pemphigoid and pemphigoid gestationis. Sera from patients with bullous pemphigoid (BP), pemphigoid gestationis (PG), and normal healthy controls (NHS), were tested in triplicate with immobilized recombinant protein. The dotted line represents the cut-off of the assay (modified from [78]).

dermal–epidermal separation in cryosections of human skin [59] and (3) serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid [60].

The histopathological examination of bullous pemphigoid patients' lesional skin reveals a sub-epidermal blister with a rich inflammatory infiltrate consisting predominantly of eosinophils and neutrophils [61]. The direct immunofluorescence microscopy reveals linear deposits of C3 and/or IgG along the dermal–epidermal junction (Fig. 4A). By indirect immunofluorescence microscopy, circulating autoantibodies binding to the skin basement membrane zone are detected in serum of bullous pemphigoid patients [62]. The use of 1 M NaCl-split normal human skin as a substrate increases the sensitivity of the indirect immunofluorescent analysis facilitating the detection of autoantibodies in 90% of bullous pemphigoid patients [63, 64] (Fig. 4B). However, indirect immunofluorescence microscopy does not allow differentiating the molecular target of autoantibodies. Therefore, molecular techniques like immunoblot (Fig. 5A) and immunoprecipitation assays are used to demonstrate the specificity of bullous pemphigoid autoantibodies [53, 65]. In addition, highly sensitive ELISAs for detecting circulating autoantibodies to recombinant BP180 have been developed [66–70] (Fig. 5B). More recently, ELISA systems using recombinant non-collagenous 16th A domain of BP180 and recombinant forms of BP230 were made
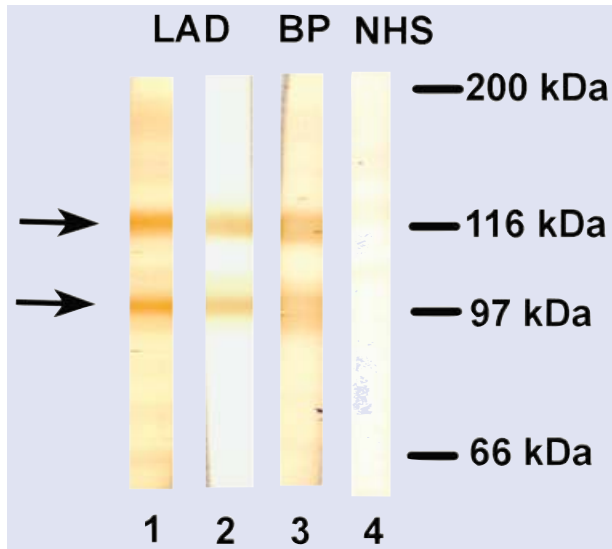
Fig. 6 Circulating autoantibodies from patients with linear IgA disease react with LAD-1 and LABD97. Concentrated supernatant of cultured keratinocytes was separated by 10% SDS-PAGE, transferred to nitrocellulose, and incubated with sera from patients with linear IgA disease (LAD; lanes 1–2), bullous pemphigoid (BP; lane 3), and healthy controls (NHS; lane 4). Subsequently, the nitrocellulose strips were incubated with HRP-labeled secondary antibodies to human IgA. Autoantibodies from patients with linear IgA disease react with both proteolytic fragments of BP180, LAD-1 (120 kD; lanes 1–2, upper arrow) and LABD97 (97 kD; lane 1, lower arrow). Serum autoantibodies from a patient with bullous pemphigoid recognize LAD-1 (lane 3). Control serum shows no specific reactivity (lane 4). Molecular weight markers are depicted on the right side of the panel.

commercially available by Medical Biological Laboratories (Nagoya, Japan) [69, 71].

Pemphigoid gestationis

Pemphigoid gestationis, previously referred to as herpes gestationis, most commonly occurs during the second or third trimester of pregnancy or in the immediate post-partum period. Skin lesions include erythematous papules as well as urticarial, eczematous, and erythema multiforme-like lesions [72]. The autoimmune response in pemphigoid gestationis is directed against BP180 and, less frequently, BP230 [73–75]. The pathogenic relevance of autoantibodies to BP180 is emphasized by the fact that passive transfer of autoantibodies from mothers suffering from pemphigoid gestationis to the foetus may induce transient skin blistering in the newborn [76, 77]. Like in bullous pemphigoid, the non-collagenous 16th A domain of BP180 has been identified as the major target of autoantibodies and is recognized by more than 80–90% of pemphigoid gestationis sera [54, 55, 78, 79].

The immunopathological hallmark of pemphigoid gestationis is linear deposition of C3 and, to a lesser extent of IgG along the dermal–epidermal junction, as detected by direct immunofluorescence microscopy. By using an immunofluorescence technique, complement-fixing circulating IgG autoantibodies, termed herpes gestationis factor, are identified in the majority of the pemphigoid gestationis sera [72] (Fig. 4C). Immunoblot and ELISA tests using a recombinant form of the 16th non-collagenous A domain of BP180 are sensitive tools for detection of autoantibodies to BP180 in patients with pemphigoid gestationis (Fig. 5) [78]. Serum levels of autoantibodies, as detected by ELISA, parallel disease activity in patients with pemphigoid gestationis [78].

Linear IgA disease

Linear IgA disease, also referred to as linear IgA bullous dermatosis, is a rare sub-epidermal autoimmune blistering disease characterized by linear IgA deposits at the dermal–epidermal junction. Linear IgA bullous dermatosis was first described in 1901 in children with non-pruriginous blistering mainly involving the genitalia [80]. However, it was not recognized as an entity separate from dermatitis herpetiformis until 1979 [81]. In a subset of patients, linear IgA disease has been shown to follow administration of drugs, most commonly after vancomycin [82, 83]. Usually, in these patients, the disease remits after the drug is discontinued. Clinically, considerable variations are seen with respect to the age of disease onset, morphology of bullous lesions and mucosal involvement. Linear IgA disease of childhood, also referred to as chronic bullous disease of childhood, is the most common autoimmune bullous disorder in children [81, 84, 85]. Cutaneous manifestations in patients with linear IgA disease include erythematous papules, urticarial plaques or vesicobullous eruptions. Lesions may appear as tense arciform bullae in a 'cluster of jewels' configuration or, less
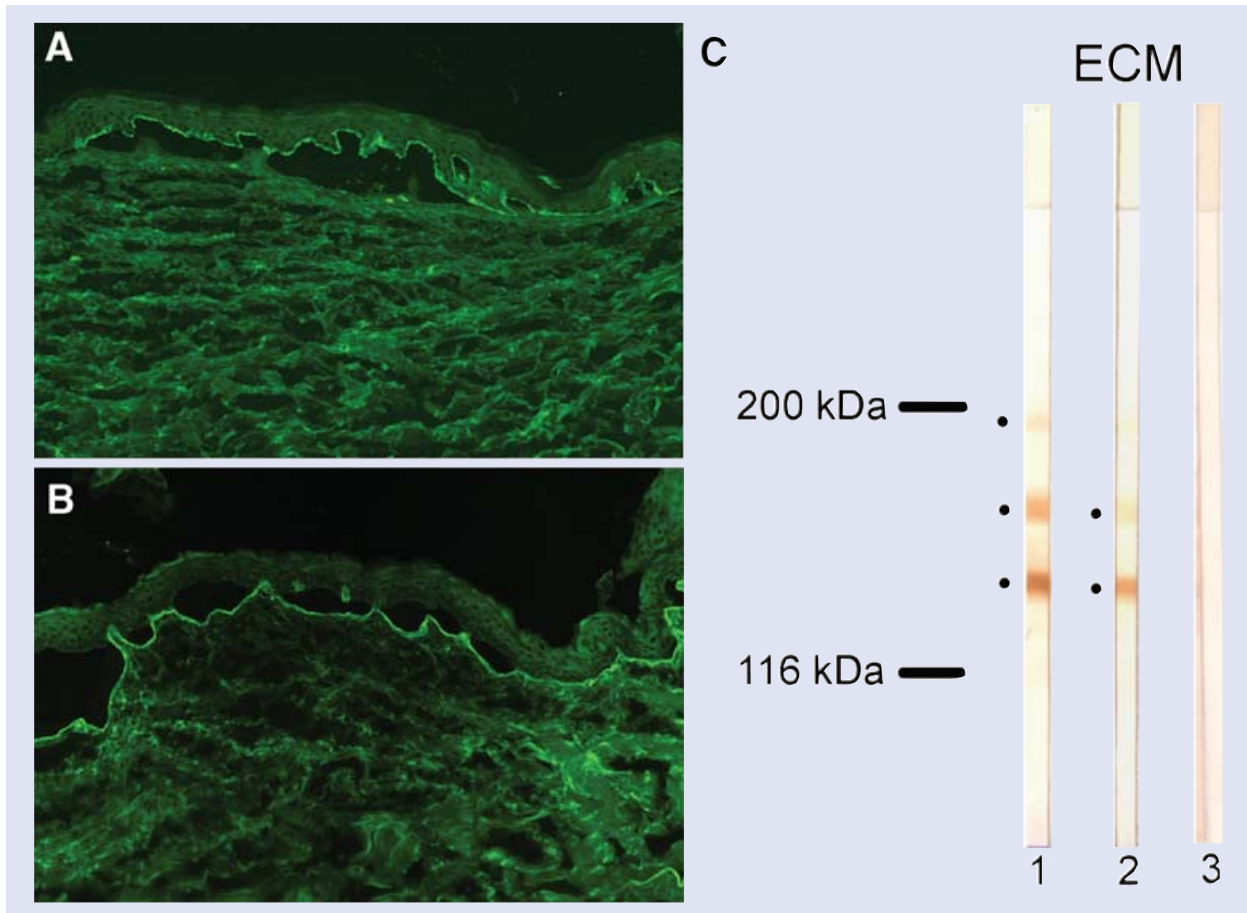
Fig. 7 Serological tests used for the diagnosis of mucous membrane pemhigoid. **(A)** Serum autoantibodies from most patients with mucous membrane pemhigoid bind to the epidermal side of the salt-split skin by indirect immunofluorescence microscopy (magnification, ×100). **(B)** In a subgroup of patients with mucous membrane pemhigoid, circulating autoantibodies show a dermal binding pattern by indirect immunofluorescence microscopy (magnification, ×100). **(C)** Extracellular matrix of cultured keratinocytes was separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and incubated with mucous membrane pemhigoid and control sera [160]. Autoantibodies from mucous membrane pemhigoid patients react with non-processed (200 kD; lane 1, upper closed circle) and processed (165 kD; lane 1, middle closed circle, and lane 2, upper closed circles) forms of the α_3-chain of laminin 5, as well as with the β_2-chain (155 kD; lane 1 and 2, lower closed circles) of laminin 5. Control serum shows no specific reactivity (lane 3). Molecular weight markers are depicted on the left side of the panel.

commonly, as grouped papulovesicles [86]. Involvement of mucosal surface is present in the majority (60–80%) of both children and adults with linear IgA disease [87, 88].

Different target antigens of the lamina lucida-type of linear IgA disease have been reported, including a 97 kD protein (LABD97) extracted from epidermis [89] and a 120 kD polypeptide (LAD-1) secreted into the medium of cultured human keratinocytes [90, 91]. Based on biochemical studies and peptide sequence analyses, it now appears that LABD97 and LAD-1 are generated as proteolytic cleavage prod-

ucts of the BP180 ectodomain [92–94]. The lamina densa-type of linear IgA disease is characterized by IgA autoantibodies recognizing dermal proteins of 180 and 285 kD [86]. Since in some patients, IgA antibodies were shown to bind to the anchoring fibrils and to specifically stain type VII collagen by immunoblotting, a new term of IgA-mediated epidermolysis bullosa acquisita was proposed for this subtype of linear IgA disease [95].

Histologically, sub-epidermal blisters and a rich inflammatory infiltrate consisting of large numbers of neutrophils, often accumulating at the papillary tips,

is characteristic of linear IgA disease. The direct immunofluorescence microscopy reveals linear deposition of IgA at the dermal–epidermal junction. By indirect immunofluorescence microscopy performed on salt-split skin, 80% of the children and 30% of the adult linear IgA disease sera label the epidermal side of the split [84]. Circulating IgA serum autoantibodies bind LAD-1 secreted into the medium of cultured human keratinocytes in 60% of the cases, as detected by immunoblot analysis [2] (Fig. 6).

Mucous membrane pemphigoid

Mucous membrane pemphigoid, previously referred to as cicatricial pemphigoid, is a chronic blistering disease of mucous membranes and skin. It may involve oral, ocular, nasal, pharyngeal, laryngeal, esophageal and anogenital mucous membranes [96]. Skin lesions appear in about 30% of patients with this disease. Mucous membrane pemphigoid is a heterogeneous disease with respect to the clinical site of involvement, the isotype of autoantibodies (IgA, IgG or the combination of IgA and IgG), the site of autoantibody binding by indirect immunofluorescence microscopy on salt-split skin (epidermal, dermal, or both), and the target antigens (BP180, laminin 5, laminin 6, $\alpha_6\beta_4$ integrin and type VII collagen) [2] (Fig. 7). About 30% of patients with the clinical phenotype mucous membrane pemphigoid reveal autoantibodies binding to the dermal side of the 1 M NaCl-split skin specific for laminin 5 [97, 98]. The pathogenic relevance of anti-laminin 5 antibodies was demonstrated by the passive transfer of anti-laminin five antibodies into mice, inducing a sub-epidermal blistering disease [99, 100]. Autoantibodies in mucous membrane pemphigoid target epitopes on both extra- and intracellular domains of BP180 [98]. In addition to the reactivity to BP180, serum autoantibodies that bind to the roof of the 1 M NaCl-split skin have been shown to target the β_4 chain of $\alpha_6\beta_4$ integrin in patients with ocular mucous membrane pemphigoid [101, 102]. These autoantibodies induced sub-epithelial cleavage in human conjunctiva cultured in vitro [103] and human buccal mucosa [104]. Rabbit antibodies generated against the immunodominant epitopes on β_4 integrin also induced bazal membrane zone separation in an *in vitro* buccal mucosa model [104]. Moreover, in patients with mucous membrane pemphigoid presenting with lesions limited to the oral mucosa, autoantibodies bound to α_6 chain of $\alpha_6\beta_4$ integrin and induced bazal membrane zone separation in normal human buccal mucosa, after 48 hrs, in organ culture [105]. Although these studies using *in vitro* cultured tissues show that autoantibodies by merely binding to their target antigens (α_6 and β_4 chains of $\alpha_6\beta_4$ integrin) induce sub-epithelial splits separation, a pathogenic role of inflammatory cells cannot be excluded.

Light microscopy studies of lesional skin or mucosa from patients with mucous membrane pemphigoid show sub-epidermal blisters and a mixed leucocytic infiltrate. Mononuclear cells, histiocytes and plasma cells dominate in lesional sites on mucosa, while eosinophils and neutrophils are also commonly seen in skin lesions. Light microscopy studies of older lesions often show fibroblast proliferation and fibrosis. Direct immunofluorescence microscopy of perilesional skin reveals continuous deposits of IgG and C3 or IgA at the basement membrane zone. Immunoreactants deposition is commonly found in perilesional mucosal biopsies as well. Splitting tissue samples with 1M NaCl facilitates the identification of circulating IgG and/or IgA autoantibodies as well as their relative distribution within the epithelial basement membranes (binding on the epidermal-, dermal- or both sides of the artificially induced blister) in 50% of the cases [2].

Anti-p200 pemphigoid

Anti-p200 pemphigoid is a recently defined autoimmune sub-epidermal blistering disease characterized by circulating and tissue-bound autoantibodies to a 200-kD protein (p200) at the interface between lamina lucida and lamina densa of the dermal–epidermal junction [106]. While the exact identity of p200 remains unknown, it has been demonstrated to be immunologically and biochemically distinct from all major autoantigens of the dermal–epidermal junction, including bullous pemphigoid antigens 180 and 230, laminin 1, 5 and 6, $\alpha_6\beta_4$ integrin, and type VII collagen [107, 108]. Clinically, most reported cases present with tense blisters as well as urticarial papules and plaques, closely resembling bullous pemphigoid or inflammatory epidermolysis bullosa acquisita [109, 110]. Histopathological examination of lesional skin biopsies shows sub-epidermal split formation and superficial inflammatory infiltrate typically dominated by

neutrophils. Immunopathologically, linear deposits of IgG and C3 are detected along the dermal–epidermal junction by direct immunofluorescence microscopy of perilesional skin. Indirect immunofluorescence microscopy of patients' sera on NaCl-split human skin demonstrates circulating IgG autoantibodies labeling the dermal side of the split. By immunoblotting, these autoantibodies recognize a 200-kD protein of human dermis [107, 109].

Epidermolysis bullosa acquisita

Epidermolysis bullosa acquisita is a chronic blistering disease of skin and mucous membranes characterized by sub-epidermal blisters and tissue-bound and circulating autoantibodies to the dermal–epidermal junction. Type VII collagen, the main constituent of anchoring fibrils, was identified as the autoantigen of epidermolysis bullosa acquisita [111, 112].

The pathogenic relevance of antibodies against type VII collagen is supported by compelling evidence: (1) Epidermolysis bullosa acquisita autoantibodies were shown to recruit and activate leucocytes ex vivo resulting in dermal–epidermal separation in cryosections of human skin [113, 114]. (2) Antibodies against type VII collagen induce sub-epidermal blisters when passively transferred into mice [115, 116]. (3) Immunization with recombinant autologous type VII collagen induces an autoimmune response to this protein resulting in a blistering phenotype closely resembling human epidermolysis bullosa acquisita [117, 118].

Epidermolysis bullosa acquisita is a clinically heterogeneous disease, which may present with an inflammatory or non-inflammatory phenotype. The first cases of a blistering disease with adult onset and features highly reminiscent of hereditary dystrophic epidermolysis bullosa were reported by Elliott more than 100 years ago [119]. The mechanobullous, non-inflammatory form of epidermolysis bullosa acquisita, was defined in 1971 and is characterized by extreme skin fragility, trauma-induced blisters and erosions localized to the extensor skin surface, healing with scars and milia [120]. In addition to the mechanobullous variant, several inflammatory subtypes of epidermolysis bullosa acquisita were described, clinically mimicking bullous pemphigoid, linear IgA disease, or mucous membrane pemphigoid [121–123]. Certain epidermolysis bullosa acquisita patients present with inflammatory phenotype at the onset of the disease with overlapping or
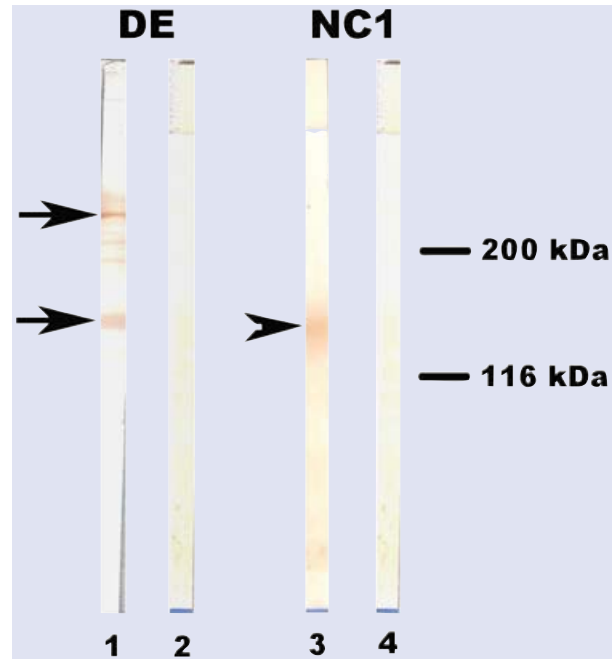

Fig. 8 Serum autoantibodies from patients with epidermolysis bullosa acquisita react with both full-length type VII collagen and with its immunodominant domain. Dermal extracts (DE) and a recombinant form of the non-collagenous (NC) domain 1 of type VII collagen were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and incubated with sera from patients with epidermolysis bullosa acquisita (lanes 1 and 3) or healthy controls (lanes 2 and 4). Sera from patients with epidermolysis bullosa acquisita react with both full-length type VII collagen (290 kD; upper arrow) and with its immunodominant NC1 domain (145 kD; lower arrow), extracted from dermis (lane 1). The same serum binds also to the recombinant form of NC1 (lane 3, arrowhead). Control serum did not react with any of the molecules (lane 2, 4). Molecular weight markers are depicted on the left side of the figure.

later evolving mechanobullous features [123, 124]. The disease typically affects adults but juvenile cases were also reported [125–127]. Epidermolysis bullosa acquisita is a rare disease occurring in approximately 5% of unselected patients with basement membrane zone antibodies [128].

Histopathological examination of lesional biopsy reveals sub-epidermal blisters associated with various degrees of inflammatory infiltrate in the upper dermis, usually concordant with the clinical presentation [129]. The classical presentation shows little inflammatory infiltrate within the dermis as opposed to the inflammatory type [130]. Ultrastructurally, the cleavage plane can be localized within the lamina
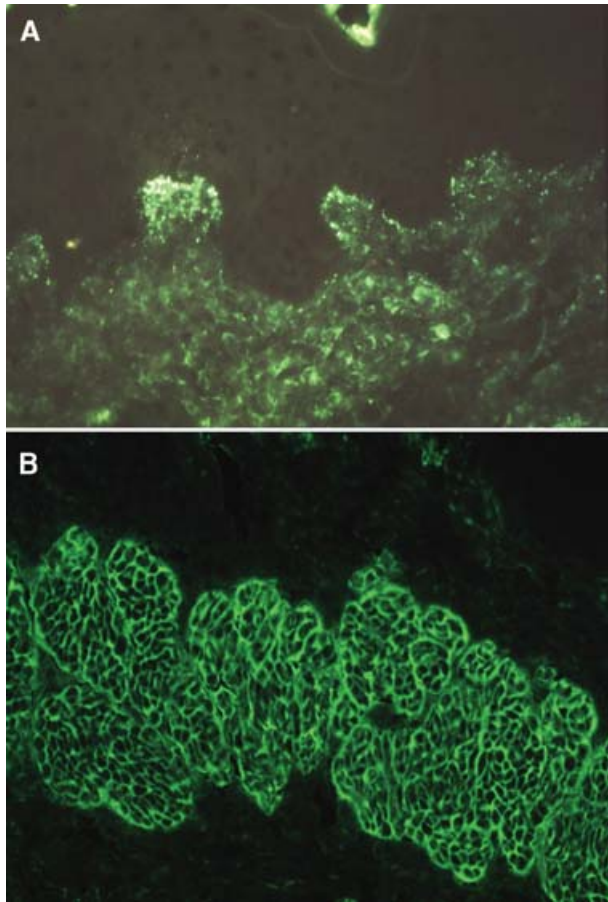
Fig. 9 IF microscopy analysis in dermatitis herpetiformis. **(A)** Direct immunofluorescence microscopy of a perilesional skin biopsy reveals granular IgA deposits in the papillary dermis (magnification, ×400). **(B)** By indirect immunofluorescence microscopy on monkey esophagus, anti-endomysial IgA antibodies are detected in serum of a patient with dermatitis herpetiformis (magnification, ×200).

lucida or sub-lamina densa regions of the dermal–epidermal junction [131]. The cleavage within the lamina lucida seems to be associated with presence of an inflammatory infiltrate rich in polymorphonuclear neutrophils [131].

The disease is immunopathologically characterized by deposition of immunoreactants at the dermal–epidermal junction by direct immunofluorescence microscopy. The IgG and/or C3 deposits were shown to localize to the sub-lamina densa region of dermal–epidermal junction by direct immunoelectron

microscopy [132]. Indirect immunofluorescence microscopy on 1M NaCl split-skin evidence autoantibodies to the dermal versant of the dermal–epidermal junction in serum of epidermolysis bullosa acquisita patients [63], which label the sub-lamina densa zone by indirect immunoelectronmicroscopy [133]. Epidermolysis bullosa acquisita serum autoantibodies recognize the 290 kD type VII collagen, or its immunodominant region, the non-collagenous domain 1, by immunoblotting with dermal extracts [111] (Fig. 8) and immunoprecipitation with keratinocyte and fibroblast extracts [134]. A sensitive ELISA for the detection of autoantibodies to type VII collagen using recombinant protein is also available [135].

Dermatitis herpetiformis Duhring

Dermatitis herpetiformis is an intensely pruritic, chronic autoimmune blistering skin disease characterized by granular IgA deposits in the papillary dermis and association with celiac disease [136, 137]. The disease was initially described by Louis Duhring, which coined the term dermatitis herpetiformis in 1884 [138]. Later studies found small-bowel changes suggestive of celiac disease in the majority of patients with dermatitis herpetiformis [139–141]. Subsequently, both diseases were shown to be associated with certain HLA haplotypes, especially with DR3 and DQw2 [142]. A major advance was the detection of IgA deposition in the papillary dermis of patients with dermatitis herpetiformis [143, 144]. Clinically, an intense pruritic rash, symmetrically distributed on the extensor surfaces including elbows, knees, buttocks, and scalp, characterizes patients with dermatitis herpetiformis. The rash is polymorphic with small blisters. These are, however, often eroded and crusted because of intense itch and scratching [145, 146]. Most patients with dermatitis herpetiformis have sub-clinical celiac disease.

Typical histopathological features of dermatitis herpetiformis are found in an early non-blistering lesion, and these consist of infiltration of granulocytes (mainly neutrophils and few eosinophils) with formation of papillary microabscesses. As the lesion develops, these microabscesses become confluent resulting in a sub-epidermal blister [136]. Histological changes in dermatitis herpetiformis are indistinguishable from those seen in the skin of patients with linear

IgA disease. Other autoimmune sub-epidermal blistering diseases may also show an inflammatory infiltrate mainly composed of neutrophils and eosinophils and may mimic dermatitis herpetiformis histopatologically, including epidermolysis bullosa acquisita, bullous pemhigoid, mucous membrane pemphigoid, and anti-p200 pemphigoid [109]. Ultrastructural studies have shown that IgA deposits are localized to the papillary dermis [147]. However, the ultrastructural localization of blister has been shown to be within the lamina lucida of the dermal–epidermal junction [148, 149].

Direct immunofluorescence microscopy of a biopsy of normal-appearing skin adjacent to an active lesion reveals granular IgA deposits in the dermal papillae [144, 150, 151] (Fig. 9A). In 15% of the cases, besides the granular IgA deposits in the dermal papillae, linear deposition of IgA autoantibodies along the dermal–epidermal junction can also be detected. Direct immunofluorescence examination of lesional skin yields frequently negative results.

The absence of circulating IgA autoantibodies to dermal components seems to be a fact in patients with dermatitis herpetiformis [152]. However, serologic markers of damaged jejunal mucosa including IgA autoantibodies to endomysium can be detected by indirect immunofluorescence on monkey esophagus [153, 154] (Fig. 9B). The autoantigens targeted by autoantibodies in dermatitis herpetiformis include tissue and epidermal transglutaminases [155–157]. Epidermal and tissue transglutaminases show cross-reactive epitopes targeted by IgA autoantibodies from patients with both celiac disease and dermatitis herpetiformis, but while IgA antibodies in patients with dermatitis herpetiformis bind to the epidermal form with high affinity, in celiac disease IgA autoantibodies preferentially recognize the tissue form of the transglutaminase family.

Recently, a disease model for dermatitis herpetiformis was developed in mice sensitized to gluten [158]. Disease induction required co-administration of gluten and pertussis toxin as an adjuvant in transgenic DQ8+ mice lacking endogenous mouse MHCII that were backcrossed on the autoimmune prone NOD mice. Approximately 15% of the immunized mice developed a sub-epidermal blistering disease after 2–5 months of gluten ingestion. Blistering was shown to be gluten-dependent and associated with granular IgA deposits in the upper dermis. However, IgA autoantibodies to endomysium, gluten, gliadin or tissue/epidermal transglutaminase have not been detected in sera of the diseased mice [158]. In an earlier study, IgA deposits from patients' skin sections were shown to recruit granulocytes *ex vivo* [159]. It is therefore conceivable that cutaneous IgA immune complexes trigger an inflammatory reaction by activating complement and granulocytes resulting in subepidermal blister formation. However, the mechanisms of IgA deposition in the skin and the specificity of deposited IgA antibodies finally are still unknown. Thus, the link between circulating IgA autoantibodies with known specificities and tissue-bound IgA that may induce blistering in dermatitis herpetiformis is still missing [8].

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