Introduction
Pemphigus comprises a group of autoimmune blistering diseases clinically characterized by flaccid blisters and erosions of the mucous membranes and/or the skin, and histologically by intra-epidermal acantholysis, i.e. loss of cell-cell adhesion. Autoantibodies circulate in the blood and deposit in the epidermis and/or mucosal epithelium of patients. There are several pemphigus subtypes. In the two main subtypes, pemphigus vulgaris (PV) and pemphigus foliaceus (PF), autoantibodies are directed against the desmosomal proteins desmoglein (Dsg3) and/or 1 (Dsg1). In the subtype paraneoplastic pemphigus (PNP), patients have an underlying neoplasm and additional antibodies directed against plakins and the protease inhibitor alpha-2-macroglobuline-like-1 (A2ML1).

The reported incidence of pemphigus ranges between 0.5-8 cases per million per year, depending on the geographic region studied. For the Netherlands the year incidence is assessed to be 2.9 per million. Pemphigus vulgaris and pemphigus foliaceus occur most frequently, while PNP is much rarer but has higher mortality. Overall, the mean age of disease onset ranges between 40-60 years, although adolescents, children and the elderly may also be affected. Up to-date around 500 PNP cases have been described worldwide, since its first description in 1990. It comprises 3-5% of all pemphigus cases (Dr. H. Pas, personal communications). The underlying neoplasm is most often lymphoproliferative in nature, such as non-Hodgkins lymphoma, thymomas and leukemia. Sarcomas and other solid malignancies may also be found. In addition benign lymphoproliferative diseases may be underlying, such as Castleman’s disease, which is most prevalent in young-adults and children with PNP.

Accurate diagnosis of any disease is important, and for PNP this may be challenging. Therefore, this thesis hopes to contribute to improve the diagnostic approach of PNP. In addition, the exact mechanisms that lead to blister formation in pemphigus are still widely debated. Therefore, in this thesis several studies on pemphigus patient skin and mucosa have been performed to get a better understanding of pemphigus pathogenesis.

**Pemphigus subtypes**

**Pemphigus vulgaris**

Patients with PV develop flaccid blisters and erosions of the mucosal epithelium (figure 1a-b) with or without skin involvement. The oral mucosa, most often the hard and soft palate, the buccal and gingival mucosa but also the pharynx and more rarely the larynx and esophagus, are affected. In addition genital mucosa and less often conjunctival mucosa may be involved. Skin involvement may include the scalp, face, upper trunk, flexures and extremities. For all pemphigus subtypes in the active disease phase, blisters can be induced on clinically unaffected skin or mucosa, by applying mechanical friction such as by rubbing. This is called the direct Nikolsky’s sign, and is often used by clinicians to monitor disease activity. Without treatment the disease course is progressive and may lead to death due to infections caused by the loss of skin and mucosal barrier function, or due to starvation as the painful oral lesions impair food intake.

The clinical phenotype is determined by the autoantibody profile. Patients with antibodies against Dsg3 have only mucosal involvement (mucosa dominant PV: mdPV) while patients with antibodies against both Dsg3 and Dsg1 have mucosal and skin involvement (mucocutaneous PV: mcPV). The histology of lesional pemphigus vulgaris mucosa and skin is characterized...
by suprabasal acantholysis (figure 2b), while the immunopathology is characterized by IgG depositions along the epidermal and epithelial cell surfaces (ECS pattern), often in a clustered pattern. Interestingly these depositions are present in both affected mucosa as well as unaffected skin.

**Pemphigus foliaceus**

Patients with PF develop flaccid blisters and scaly, crusty erosions of the skin. 1 Typical locations include the seborrheic regions of the scalp, face, and upper trunk, but lesions may also be more widely distributed (figure 1e-f). Mucosal tissue is clinically unaffected. Autoantibodies are directed against Dsg1, and the histology is characterized by subcorneal acantholysis (figure 2a). Immunopathology shows ECS IgG depositions in the skin but also the mucosa, and similar to PV also in a clustered pattern.

**Pemphigus erythematosus**

Pemphigus erythematosus is a subtype of PF, with the same histology and autoantibody specificity, but with different distribution of lesions and immunopathology. Lesions are located in the seborrheic areas, however with also a characteristic involvement of the malar region (figure 1d). This distribution resembles the butterfly-rash seen in systemic lupus erythematosus, thus the name pemphigus erythematosus (PE).

In addition to IgG depositions along the ECS, immunopathology of PE skin may show IgG depositions along the basement membrane zone (BMZ), similar to the ‘lupusband’ seen in lupus erythematosus, in approximately 60% of cases. Whether or not other proteins are components of this lupusband in PE is unknown, and as previous studies investigating the nature of the ECS IgG depositions in PF and PV have led to better understanding of pemphigus pathogenesis (see pathogenesis section), in chapter 2 we investigate the nature of the ‘lupusband’ in PF patients with a PE phenotype.

**Paraneoplastic pemphigus**

PNP is sometimes referred to as Paraneoplastic Autoimmune Multiorgan Syndrome (PAMS), because next to the mucous membranes and the skin, other organs such as the lungs may be affected, and because the histological hallmark for pemphigus, i.e. intraepidermal acantholysis, is not always present in PNP. 22,23 The most characteristic clinical feature of PNP, is a painful severe oral stomatitis, with hemorrhagic crusts and erosions of the intra-oral mucosa, extending to include the vermilion border of the lips (figure 1c). Conjunctival and genital mucosa may also be involved. Cutaneous manifestations range from flaccid to tense blisters and erosions as seen in pemphigus vulgaris and bullous pemphigoid, painful erythema and skin detachment as seen in toxic epidermal necrolysis (figure 1g), targetoid lesions as seen in erythema multiforme, and lichenoid papules and plaques as seen in lichen planus (figure 1h), or the variable manifestations of graft versus host disease, but may also be absent in a subset of patients. The distribution typically involves the face, trunk and extremities, but may also include palms and soles, which distinguishes it from the other pemphigus variants. 5,9,10,22,23 A subset of patients, ranging from 8-93%, may develop shortness of breath or even respiratory failure, due to bronchiolitis obliterans. 24-26 Histological features of PNP vary, including intra-epidermal acantholysis, subepidermal blistering, interface dermatitis (figure 2c) and keratinocyte apoptosis and necrosis. 5,9,10,22,23 The autoantibody response in PNP is directed against multiple antigens found in skin and mucosa,
including the proteins of the plakin family (such as envoplakin, periplakin, desmoplakin and BP230), the protease inhibitor alpha-2-macroglobulin-like 1 protein (A2ML1) and the desmosomal cadherins desmoglein 3 and less often desmoglein 1. Immunopathology shows IgG depositions along the ECS and sometimes also along the BMZ. In a small subset of PNP patients, often with lichenoid skin lesions, no antibodies are detected, probably because the cellular autoimmune response, and not the humoral, dominates in these patients with ‘lichenoid PNP’.  

Figure 1. Clinical manifestations of pemphigus. Erosions of the palatal (a) and buccal (b) mucosa and the lateral sides of the tongue in a patient with pemphigus vulgaris. Hemorrhagic crusts and erosions extending to include the vermilion border of the lips in a patient with paraneoplastic pemphigus (c). Erythematous erosive, scaly and crusty plaques involving the malar region (d) characteristic for pemphigus erythematosus. Multiple crusted and scaly, erosive papules on the back (d, detail in e) of a patient with pemphigus foliaceus. Extensive erosions and detached skin on the chest of a patient with paraneoplastic pemphigus (g). Lichenoid erythematous papules and plaques on the back of a patient with paraneoplastic pemphigus (h).
The diagnosis of pemphigus
The diagnosis of all pemphigus subtypes is based on the combination of clinical and histological features and the demonstration of autoantibodies. Several tools exist to demonstrate the presence of these autoantibodies.

Direct immunofluorescence microscopy of tissue
IgG autoantibody depositions (or any other immunoglobulin isotype) in patient skin or mucosa can be visualized by direct immunofluorescence microscopy (DIF). This technique makes use of patient skin or mucosa biopsies, which are snap-frozen and sectioned, mounted on glass microscope slides and air-dried. Sections are then incubated with fluorochrome conjugated anti-human IgG antibodies, and visualized under an immunofluorescence microscope. The staining patterns generally reveal IgG depositions in a clustered-ECS pattern for most pemphigus subtypes (figures 3a and 3c), while concurrent BMZ IgG depositions may be seen in PE and PNP (figure 3b), as described earlier.

Serological assays
IgG autoantibodies (or any other immunoglobulin isotype) circulating in the blood of patients can be detected by indirect immunofluorescence (IIF), immunoprecipitation, immunoblotting or ELISA.

Indirect immunofluorescence microscopy
In this technique patient serum is incubated with air-dried sections of various substrates. If the autoantigen is present in the used substrate, the IgG autoantibodies (or other isotypes) in the serum will bind to the section. This first incubation is followed by a second incubation step using fluorochrome conjugated anti-human IgG antibodies. Sections are then viewed under an immunofluorescence microscope. The 2-step or indirect nature of this technique, in contrast to the 1-step used in DIF, has lead to the term indirect immunofluorescence. Substrates frequently used are monkey esophagus to detect circulating anti-ECS or –BMZ autoantibodies, and rat bladder to detect circulating anti-plakin autoantibodies (figure 3d). In addition, salt split skin,
which has a consistent lamina lucida split induced by incubation with 1M NaCl, is frequently used for the detection and further specification of circulating BMZ autoantibodies. 29,30

Immunoblotting
In contrast to DIF and IIF, immunoprecipitation (IP), immunoblotting (IB) and ELISA are serological techniques by which the specificity of autoantibodies can be determined more precisely, based on the size of proteins recognized by the patient serum (figure 3e). Immunoblotting involves the incubation of patient serum on a membrane containing keratinocyte protein extracts. Prior to being transferred to the membrane, the proteins have been chemically reduced, destroying conformational epitopes, and the proteins have been separated based on size using gel electrophoresis. The serum incubation step is followed by secondary and sometimes tertiary antibody incubation steps, and the autoantibodies are then visualized using enzyme-conjugated antibodies that convert a chemical substrate into a visible colour. Antigen containing extracts can be prepared from cultured keratinocytes or from human epidermis. When using cultured cells the differentiation stage of the keratinocytes partially determines which autoantibodies can be detected. In addition, the detection of autoantibodies by IB depends on whether or not the autoantibodies bind to conformation sensitive epitopes that are destroyed during the IB process. IB is therefore generally not used to detect anti-desmoglein antibodies in the diagnosis of pemphigus as the autoantibodies in are mainly directed against conformational epitopes. In contrast, IB is suitable for the detection of plakins in the diagnosis of PNP, in which linear (but also conformational) epitopes are recognized. 32

Enzyme-linked immunosorbent assay
In ELISA, a plate, coated with the specific autoantigen of interest, usually in its native conformation, is incubated with patient serum. This is followed by enzyme-conjugated secondary antibody incubation step, and finally the amount of bound secondary antibody is measured by incubation with a substrate that can be converted by the enzyme in a coloured product. The measured colour intensity correlates to the amount of autoantibodies present in the serum. For PV and PF, the development of the Dsg1 and Dsg3 ELISA techniques has proven to be of great diagnostic value, because the conformational epitopes are retained in this technique, and therefore the specificity of the autoantibodies can be determined accurately. Also, the quantification of autoantibody titers is possible, enabling monitoring of the disease course. 14,29,30,33,34 Recently an envoplakin-ELISA has been developed for the diagnosis of PNP. 35 Although its specificity and sensitivity was originally stated to be high by its developers, no studies have been performed comparing this ELISA to the other available serological techniques.

Immunoprecipitation
In this technique, patient serum is incubated with IgG-binding beads such as protein G sepharose. The beads are then incubated with a keratinocyte extract that contain the suspected autoantigen(s). The IgG on the beads will bind the autoantigens and after washing, the bound antigens are eluted from the beads and analysed. The antigens are separated based on molecular size, using gel electrophoresis, and visualized using either western blotting techniques (figure
3e) or autoradiography, depending on whether or not radioactively labeled keratinocyte extracts were used. As in IB, for IP the differentiation stage of the keratinocytes should be tailored to the autoantigen of interest. For example, an important and unique autoantigen in PNP is A2ML1, which is only expressed in differentiated keratinocytes. Therefore, for PNP, it is essential that the keratinocytes used are adequately cultured to reach the required grade of differentiation. An important feature distinguishing IP from IB is that in IP, the autoantibodies are exposed to the native or conformational epitopes of the autoantigen, while in IB the antigens generally need to be extracted with a harsh soap that destroys conformational epitopes leaving only linear epitopes available for binding. Therefore IP is suitable for the detection of autoantibodies directed against conformational epitopes. As A2ML1 is a protein which contains multiple disulphide bonds that secure its conformation and as it has been show that anti-A2ML1 autoantibodies in PNP mainly recognize conformation sensitive epitopes, we hypothesize that IP is an important diagnostic tool in PNP. This has however never been proven against the other available serological techniques.

Figure 3. Autoantibody detection in pemphigus.

Direct immunofluorescence microscopy shows IgG depositions (green) along the epidermal and epithelial cell surfaces in the skin (a) and mucosa (c) of a patient with pemphigus foliaceus. Note the dotted or clustered deposition pattern in the lower cell layers, as oppose to the smoother linear depositions in the higher layers. Concurrent IgG depositions along the basement membrane zone (b) may be found in the skin of patients with paraneoplastic pemphigus or pemphigus erythematosus. Nuclei are depicted in blue. Indirect immunofluorescence microscopy shows that serum of patients with paraneoplastic pemphigus may contain IgG that binds to ratbladder urothelium (d). Immunoblot (e, left lane) may be used to detect circulating anti-plakin autoantibodies, while immunoprecipitation (e, right lane) may detect additional anti-A2ML1 antibodies.
PNP, a diagnostic challenge
Of all pemphigus subtypes, the diagnosis of PNP is most challenging, due to its rarity and the large variety of clinical and histological manifestations. Over the years several attempts at formulating the diagnostic criteria for PNP have been made, and the detection of specific autoantibodies are considered one of the major criteria. \(^4,5,10,22,36\) It is not known which of the above-mentioned techniques is best for autoantibody detection in PNP. **Therefore in chapter 3 we compare the sensitivity and specificity of an array of serological laboratory techniques in the diagnosis of PNP. In our comparison we include a self-developed non-radioactive immunoprecipitation assay. In addition, in chapter 4 we zoom in on the different DIF and IIF staining patterns and their usefulness in diagnosing PNP.**

Pemphigus vulgaris and foliaceus targets: desmosomal cadherins

**Desmosome structure**
Desmosomes are junctional protein complexes (figure 4) that mediate intercellular adhesion. They are present in all stratified epithelium but also in other mechanical stress-bearing tissues such as myocardium. Desmosomes are essential in maintaining tissue integrity, and loss of desmosomal function results in tissue fragility. \(^37-40\) As visualized by electron microscopy, desmosomes can be divided in 3 regions: the intercellular dense midline, the cytoplasmic outer dense plaque and cytoplasmic inner dense plaque. The dense midline is composed of desmosomal cadherins: desmogleins and desmocollins. \(^41,42\) These are transmembrane glycoproteins that provide intercellular adhesion by binding to the extracellular N-terminal of their opposing counterpart. At their cytoplasmic C-terminal, cadherins bind to the proteins of the armadillo family: plakoglobin and plakophilin, which form the components of the cytoplasmic outer dense plaque. Besides mediating adhesion within the desmosomal protein complex, plakoglobin and plakophilin also play a role in regulating desmosome assembly. \(^43-51\) These proteins in turn bind to desmoplakin, of the plakin family. Desmoplakin's tail, situated in the cytoplasmic inner dense plaque, directly binds to the cytoskeletal intermediate filaments.
Figure 4. Desmosome structure.
A schematic illustration of desmosome structure, showing the location of desmosomal cadherins, plakins, armadillo proteins and intermediate filaments. An electron microscopy picture of a desmosome is shown to the left, and at the bottom the structure of adherens junctions is displayed. Drawing by M. F. Jonkman.

**Desmosomal cadherins**

The desmosomal cadherins, desmoglein and desmocollin, are part of the cadherin family of proteins. Other proteins in this family include the classical cadherins such as the adherens junction protein E-cadherin. Cadherins are transmembrane proteins that all have an extracellular domain (EC) made up of a variable number of 110-amino acid motifs. The main function of the extracellular domain is adhesion, which is dependent on calcium, as calcium binding induces the correct conformation. The transmembrane domain of cadherins are followed by an intracellular anchor domain (IA) which is a binding site for plakoglobin and other catenins. The cadherin subtypes vary in the rest of their cytoplasmic domains, resulting in different protein partner binding properties, which in turn leads to functional variation. The cytoplasmic domain of desmogleins is distinguishable from that of other cadherins by its unique proline rich linker domain (L), a repeated unit domain (RUD) containing a variable number of a 29-amino acid motif, and a desmoglein terminal domain (DTD). 39
Four isoforms of desmogleins have been identified, Dsg1-4, each with 5 ECs (EC1-5), and a variable number of cytoplasmic RUD-motifs. As stated earlier, Dsg1 and Dsg3 are the main autoantigens in pemphigus, and for both Dsg1 and Dsg3, EC1 and EC2 are the main targeted epitopes in PV and PF, while in PNP a significant proportion of Dsg3 antibodies are additionally directed against EC3, EC4 and to a lesser extent against EC5. 52,53

Desmoglein 1
Desmoglein 1 is 160kDa in molecular mass. In skin (figure 5a), Dsg1 is expressed in all cell layers, and expression levels follow a gradient, with highest expression in the more differentiated superficial cell layers, and lower expression towards the undifferentiated basal cell layers. In the oral mucosa (figure 5d), Dsg1 is expressed evenly in all layers except the basal cell layers, where it is absent. 39,54,55 The distribution of Dsg1 is altered in patients with anti-Dsg1 antibodies, where it is clustered (figure 5g).

Besides its adhesive role in maintaining tissue integrity, Dsg1 has been allotted other functions, such as regulating EGFR-signalling and promoting keratinocyte differentiation. 39,56,57 In addition it has been found to be a target for caspase degradation, and to have a role in regulating apoptosis. 58

Desmoglein 3
Dsg3 is 130kDa in molecular mass. It is expressed in the basal and first few suprabasal cell layers of the epidermis (figure 5b), and is absent in the superficial epidermis. In the oral mucosa, Dsg 3 is expressed in all cell layers (figure 5e), and at much higher levels than that of Dsg1. Similar to Dsg1, the distribution of Dsg3 in patients with anti-Dsg3 antibodies is clustered (figure 5h). Like Dsg1, Dsg3 also has other functions besides adhesion, such as driving proliferation and upregulating EGFR signaling. 56
Figure 5. Desmoglein 1 and desmoglein 3 distribution in skin and mucosa. Immunofluorescence microscopy shows that in normal human adult skin, desmoglein 1 (Dsg1) is located smoothly along the cell surfaces of all epidermal layers, with increased expression in the upper layers (a), while Dsg3 (b) is expressed in the lower layers. A Dsg1/Dsg3 overlay is shown in (c). In normal human mucosa Dsg1 is expressed in all but basal epithelial layers (d), while Dsg3 is expressed in all layers (e). A Dsg1/Dsg3 double staining is shown in (f). The dashed line indicates the basement membrane zone. In pemphigus foliaceus skin, Dsg1 distribution is often clustered (g), and in pemphigus vulgaris skin, Dsg3 is often clustered (h). Nuclei are depicted in blue.

Desmosomal assembly and disassembly
Desmosomal proteins are synthesized in the cytoplasm and transported to the cell membrane. The exact transportation mechanism for Dsg1 and Dsg3 are unknown, but for Dsg2 and Dsc2 it has been shown that the motor proteins kinesins mediate their transportation along microtubules.
to the cellular membrane. For Dsc2 but not Dsg2, kinesin mediated trafficking depends on plakophilin 2. Dsc and Dsg furthermore differ in their transportation kinetics, with Dscs initiating desmosome assembly, while Dsgs arrive later to stabilize the complex. Similar mechanisms may account for the transportation of Dsg1 and -3 to the cell membrane, but this has not yet been studied.

During desmosomal assembly, desmosome size is regulated by several mechanisms. Firstly, the interaction of desmosomal cadherins with their binding partners differs per isoform, and is thought to account for their different contributions to desmosome size. Dsg 1 is thought to interact with 2-6 molecules plakoglobin (PG), while this Dsg-PG ratio is less for Dsg3. Thus, desmosomes containing more Dsg1 may be larger than those with more Dsg3. Secondly, the armadillo proteins plakophilin and plakoglobin regulate desmosome size. In line with these findings, recent studies of PF patient skin have shown that both plakoglobin and Dsg1 distribution are disrupted, and that desmosomes are smaller. Whether desmosomal changes occur in the mucosa of these patients is unknown.

In vitro studies have shown that desmosomal assembly is dependent of calcium, as desmosomes are absent in low-calcium culture medium (< 0.1mM), while increase in calcium concentration induces desmosome formation. Calcium binds to desmosomal cadherins to induce an adhesive conformation. In addition, two types of desmosomes are thought to exist: Calcium-independent desmosomes, which are hyperadhesive, and calcium-dependent desmosomes. The normal epidermis is thought to contain mainly calcium-independent hyperadhesive desmosomes, while in cells cultured to sub-confluence and early confluence stages, and in cells at wound edges, desmosomes are thought to be calcium-dependent. The phosphorylation of desmosomal components may influence hyperadhesion, possibly due to altered organization of the desmosomal proteins. Besides regulating desmosomal assembly and size, plakophilin and plakoglobin are needed for desmosomal hyperadhesion.

These insights on the different adhesive states of desmosomes, in vivo and in vitro, have important implications for the interpretation of in vitro data in pemphigus research, as in vivo data might not readily represent the conditions in human skin. We therefore advocate the use of human tissue in pemphigus research. Therefore in chapters 2, 5 and 6 we make use of patient skin and mucosa biopsies to get more insight in desmosomal alterations and pemphigus pathogenesis.

The exact mechanism involved in desmosome degradation or disassembly is unknown. Whole desmosomes may be internalized and degraded, as is suggested by an electron microscopy study of a wound healing experiment. On the other hand, desmosomes may be disassembled through the internalization and degradation of individual desmosomal components. For the latter less evidence is available due to technical difficulties in determining whether or not desmosomal components are truly in- or outside of desmosomes.

**Pemphigus pathogenesis**

It is well established that anti-Dsg1 and –Dsg3 antibodies are necessary and sufficient to induce acantholysis in pemphigus. In addition, it has been proven that the antibody profile dictates whether skin or mucosa is affected, as explained by the desmoglein compensation theory. The exact cellular mechanism by which antibodies induce acantholysis is, however still a matter of lively debate.
**Desmoglein compensation theory**

The desmoglein compensation theory provides an explanation for the locations of blisters in the skin and mucosa of pemphigus patients. The theory is based on the following findings. Firstly, in 1996, Amagai et al. noted that the suprabasal level of blistering seen in pemphigus vulgaris, correlated to the basal and immediate suprabasal location of Dsg3 in skin. 73 Although later studies shed more accurate light on the autoantigens in pemphigus vulgaris, this study provided the first hint that desmoglein distribution may be linked to the distribution of pemphigus lesions. In 1997, two studies showed that pemphigus patients with mucosal involvement versus mucocutaneous involvement have distinct antibody profiles. Patients with mdPV were shown to have anti-Dsg3 antibodies only, while patients with mcPV had both anti-Dsg3 and anti-Dsg1 serum reactivity. 13,14 In addition, it was already known that in PF, patients have only skin but no mucosal involvement and only anti-Dsg1 autoantibodies. In 1998 Shirakata et al. showed that the expression level of Dsg1 in the oral mucosa was significantly less than that of Dsg3. They proposed that, in PF, this is the reason for the lack of mucosal involvement, as Dsg3 is present in sufficient amounts to compensate for the autoantibody-induced loss of function of Dsg1. 54 In 1999 Mahoney et al. explained the clinical and histological locations of lesions in both PV and PF, based on their passive transfer experiments of pemphigus IgG in neonatal mice. 74 They showed that mice completely lacking Dsg3, injected with anti-Dsg1 IgG, showed more severe blisters, deep in the epidermis and also in the mucosa than the mice with normal Dsg3 expression, showing the protective function of Dsg3 in these locations. In addition they showed that for PV, both anti-Dsg1 and anti-Dsg3 were needed for epidermal blister formation. In line with these results, in 2000 Wu et al. proposed that the expression of Dsg3 in all cell layers of neonatal human skin protects the neonate from developing subcorneal blisters, when exposed to maternal PF IgG. 75 They provided evidence for this by showing that transgenic mice with ectopic Dsg3 expression in the superficial epidermis do not develop subcorneal blisters after injection with PF IgG. In conclusion, desmoglein 1 and 3 show functional redundancy, and the autoantibody-induced loss of function of one desmoglein isoform may be compensated for by another isoform. Therefore, acantholysis occurs at the location where the autoantibody targeted desmoglein isoform is expressed, providing that there is no or insufficient alternative desmoglein isoform present to compensate for the target’s loss of function. In other words, the autoantibody profile determines the involvement of skin or mucosa and the histological level of blistering. Patients with only anti-Dsg3 antibodies show suprabasal acantholysis of the mucosa, as the amount of Dsg1 in the mucosal basal layers is insufficient to compensate. In contrast, patients with anti-Dsg1 antibodies have subcorneal blisters but no clinical signs of mucosal involvement, due to the lack of Dsg3 to compensate for the loss of Dsg1 in the superficial epidermis, and the strong compensatory expression of Dsg3 in mucosa. Patients with both anti-Dsg1 and Dsg3 antibodies have blistering of both skin and mucosa, as compensatory mechanisms are compromised in both tissues. Here the suprabasal level of blistering is however not explained by the location of Dsg1 or Dsg3, but probably by the fact that the basal cell layers first encounter IgG that diffuses from the dermis or lamina propria upwards.
Desmoglein compensation may not be absolute: insights from uninvolved tissue

For mdPV it has been shown that despite the presence of anti-Dsg3 IgG depositions, and the clustered distribution of Dsg3, the skin of mdPV patients shows no further pathology, even at an ultrastructural level. Does the same hold true for the clinically unaffected mucosa of PF patients? Studies have suggested that the effects of anti-Dsg3 IgG depositions may significantly differ from those of anti-Dsg1 IgG. More specifically, van der Wier et al. showed that in the skin of N+ patients with anti-Dsg1 antibodies, desmosomes are smaller and reduced in number. In addition, Oktarina et al. showed that in the areas of heavy Dsg1 clustering, and IgG deposition, intercellular widening was seen in PF and mdPV skin. Notably this widening was present in the lower epidermal layers of PF skin, where one would not expect any pathology, as the hallmark for PF is subcorneal acantholysis. These signs of pertubated cell-cell apposition and desmosome hypoplasia were not observed in the skin of patients with only Dsg3 antibodies. This could imply that, in contrast to the skin of mPV patients, the healthy appearing mucosa of PF patients may be subjected to pertubated cell-cell apposition and desmosome hypoplasia. Studies on endemic PF mucosa have indeed shown intercellular widening and desmosomal changes. Furthermore, already in 1983 Hietanen et al. also showed intercellular widening of the lower epithelial cell layers of uninvolved mucosa of PF, PE and PV patients. However, these studies were not quantitative in nature and did not address whether the ultrastructural changes were related to IgG depositions, Dsg1 clustering or the alteration of other junctional proteins. Therefore, in chapter 5 we investigated the ultrastructure in the mucosa of patients with non-endemic PF, in relation to Dsg1 clustering, the distribution of other junctional proteins, and IgG depositions.

Hypotheses on the cellular mechanism of acantholysis

Several hypotheses exist on the cellular mechanism by which pemphigus autoantibodies induce loss of intercellular adhesion.

- Steric hindrance

Several observations have led to the hypothesis that upon binding, anti-Dsg antibodies directly interfere with the adhesive interface of desmogleins. This is based on the following observations. Firstly, the majority of PV and PF patients, have anti-Dsg antibodies that target the EC1 and EC2 domains. These domains are both involved in desmoglein trans-adhesion. In addition, when selectively removing these EC1 and EC2 specific autoantibodies from purified PV and PF IgG, the remaining IgG loses its acantholytic potential when injected in neonatal mice. Furthermore, atomic force experiments in a cell-free model have shown that anti-Dsg3 antibodies reduce the trans-adhesive force between desmogleins. Finally, electron microscopy studies of lesional PV skin have revealed the presence of half-desmosomes on acantholytic cells, suggesting that by binding to the trans-adhesive interface of Dsgs, autoantibodies sterically hinder desmoglein trans-adhesion, causing desmosomes to split in half. Besides trans-adhesional steric interference, a recent study has shown that also the cis-adhesional interface of Dsg 3 may be obstructed in PV. A subset of pemphigus patients has autoantibodies directed against other domains than the adhesive EC1 and EC2 domains, and adsorption of EC1 specific autoantibodies from PV IgG only partially reverses its pathogenicity. In addition, studies have shown desmosomal changes...
other than half-desmosomes; such as a reorganization of the cytoplasmic desmosomal plaque in a PV mouse model and reduction in desmosome size as well as clustering of desmosomal components in pemphigus patient skin. These findings suggest that, besides steric hindrance, other mechanisms play a role in acantholysis.

- Interference in desmosome assembly or disassembly

For proper assembly of desmosomes, an adequate pool of desmosomal components should be available to be incorporated into the desmosome. Several studies have indicated that PV and PF IgG may interfere with the availability of this pre-desmosomal or non-desmosomal protein pool. In PV and PF patient skin biopsies, a re-organization of Dsg3, Dsg1 and plakoglobin has been found. Instead of a smooth distribution along the epidermal cell surface (ECS) these proteins are clustered along the cell surfaces. These clusters co-localize with intraepidermal IgG depositions, and which Dsg isoform has been affected correlates to the antibody profile of patients; ie Dsg1 for PF and Dsg3 and/or Dsg1 for PV. Several studies have interpreted these clusters to be entire desmosomes. However, in patient skin these clusters have been shown do not contain all desmosomal components, as desmoplakin did not co-localize with them. Furthermore, clustering was dependent on the bivalency of IgG, as Fab fragments were not able to induce this clustering in an evivo human skin explant pemphigus model. Therefore it is more likely that these clusters represent non-desmosomal pools of Dsgs and plakoglobin, that have been sequestered or cross-linked into clusters by anti-Dsg IgG. In line with this, electron and immunofluorescence microscopy studies of PV patient skin and cultured keratinocytes have shown autoantibody depositions not only on the desmosomes, but also along the cell membrane without desmosomal structures. However in another study only desmosomal autoantibody depositions were found. Further studies have shown that desmosome size and number is reduced in lesional and Nikolsky-positive PF and mcPV skin. Therefore, the sequestration of non-desmosomal Dsg and plakoglobin into clusters may render these molecules unavailable for incorporation into desmosomes, resulting in smaller and less desmosomes (Figure 6). Similar clustering of Dsg and IgG has been found in studies using cultured keratinocytes incubated with PV IgG. Evidence supporting the depletive effects of anti-Dsg IgG on desmosomal assembly is mainly obtained from in vitro experiments with cultured keratinocytes and in vivo mouse models. Incubation of cultured keratinocytes with pemphigus vulgaris IgG or monoclonal anti-Dsg3 antibodies first leads to a reduced amount of Dsg3 in the triton soluble pool of cell membrane proteins. After several hours, also the tritons insoluble pool is depleted of Dsg3. Mice exposed to anti-Dsg3 IgG show similar effects. The triton soluble pool of Dsg is interpreted as being composed of non-desmosomal membrane bound Dsg, while the triton insoluble pool is thought to be composed of desmosomal Dsg. Therefore, these findings support the hypothesis that in pemphigus, anti-Dsg IgG interferes with desmosomal assembly by binding to and depleting the pool of pre-desmosomal components. There is evidence that the depletion of desmogleins from desmosomes occurs via autoantibody induced endocytosis, and subsequent degradation of Dsgs. In addition, in PV the less differentiated, lower epidermal layers may be more prone to this depletion. Most studies have predominantly focused on the depletive effects of anti-Dsg3 autoantibodies.
For the depletive effects of anti-Dsg1 autoantibodies there is substantially less evidence. In vivo, the smaller desmosomes in the skin of PF patients can be viewed as indirect evidence of the depletive effects of Dsg1 IgG in PF. However, direct proof of Dsg1 depletion from desmosomes in PF patient skin is lacking. Electron microscopy studies that investigate the ultrastructure of pemphigus patient tissue have provided us with improved insights in pemphigus pathogenesis. Besides the aforementioned shrinkage of desmosomes in N+ PF patient skin, intercellular widening has been observed in the areas where Dsg1 clusters are most abundant. This widening could also be reproduced in vitro in cultured keratinocytes exposed to anti-Dsg1 PF IgG. Despite these insights, the ultrastructure of the Dsg1 clusters in PF skin is yet unknown. Therefore, in chapter 6 we use immunoelectron microscopy on PF patient skin, to determine the ultrastructural fate of Dsg1 and whether or not desmosomes are depleted of Dsg1, in PF pathogenesis.

Figure 6. Proposed mechanism of desmosomal depletion and acantholysis in pemphigus.
Under normal circumstances, non-desmosomal desmogleins of various isoforms (each colour represents an isoform) are continuously being built in and discarded from desmosomes (A). In pemphigus, anti-Dsg autoantibodies bind to non-desmosomal desmogleins, preventing their incorporation into desmosomes. When only one of the Dsg-isoforms is targeted, desmosomes are selectively depleted of these isoforms (B). When both Dsg isoforms are targeted by the autoantibodies, no compensation is possible, desmosome assembly is halted, desmosomes ‘melt’ away, and acantholysis occurs (C). The figure is taken from Oktarina et al.18
Multiple signaling pathways have been implicated in pemphigus pathogenesis, and pharmacologic intervention in such pathways may provide us with new therapeutic options for pemphigus. These pathways include the EGFR (epidermal growth factor receptor), mTOR (mammalian target of rapamycin) and p38MAPK (p38 mitogen activated protein kinase) signaling pathways. Drugs that target the EGFR and mTOR pathways are currently already being used as effective therapies for other diseases. EGFR-inhibitors are used to treat lung carcinoma patients and sirolimus, an inhibitor of the mTOR pathway, has proven to be effective in preventing organ rejection in kidney transplantation patients. In addition, inhibition of the p38MAPK pathway has been attempted in the treatment of inflammatory diseases such as rheumatoid arthritis. However, the toxicity profiles of these p38MAPK-inhibitors are a cause for concern.

What is the evidence for the role of the EGFR, mTOR and p38MAPK signaling pathways in pemphigus pathogenesis? Both in vitro studies with cultured keratinocytes that are incubated with PV IgG, as well as in vivo mouse models in which mice are injected with PV IgG have shown that IgG induces the activation of EGFR signaling, as shown by the upregulation of downstream signaling molecules such as c-myc. In addition, inhibitors of EGFR signaling prevent Dsg3 endocytosis and acantholysis in these models. Furthermore, the phosphorylation of p38MAPK becomes increased in patient skin and in mice treated with both PV and PF IgG, and the inhibition of p38MAPK also prevented acantholysis in vitro and in vivo. Similarly, the mTOR-signaling pathway, which acts downstream of EGFR, may play a role in acantholysis, and the inhibition of mTOR-signaling using sirolimus has been proposed as a new therapy for pemphigus.

mTOR
mTOR is a serine/threonine protein kinase. It is a cytoplasmic protein that binds to other proteins to form 2 types of complexes: TOR complex-1 (TORC1) and TORC2. This introduction will focus on TORC1, as this is targeted by sirolimus. Besides mTOR, TORC1 is composed of the proteins raptor and mLST8. mTOR can be activated via several growth factors which bind to growth factor receptors on the cell membrane. These factors include insulin, insulin like growth factor (IGF) and the epithelial growth factor (EGF). In addition, stress, hypoxia and a shift in nutrient availability can trigger the mTOR pathway. Binding of the growth factors to their receptors results in the activation of Phosphatidylinositol 3 kinase (PI3K), Akt, Tuberous Sclerosis Complex (TSC) and Ras homolog enriched in brain (Rheb) signaling cascade. This results in the phosphorylation and activation of mTOR and several of its TORC1 binding partners, and the regulation of several downstream signaling molecules including S6K1 and 4EBP1. Phosphorylation of mTOR at Serine-2448 is regarded as a marker for active mTOR signaling, and this signaling pathway is a crucial regulator of cell growth, survival, proliferation, vesicular trafficking and cytoskeletal organization.
mTOR and pemphigus pathogenesis
An increase in mTOR ser-244 phosphorylation was observed in the basal keratinocytes of neonatal mice skin, upon injecting these mice with PV IgG. Furthermore, the inhibition of the mTOR-signaling pathway by pretreating the mice with intradermal sirolimus, inhibited acantholysis. This suggests that the activation of the mTOR-signaling pathway precedes acantholysis in pemphigus pathogenesis. In line with this, two case reports have been published that advocate the use of systemic sirolimus in the treatment of PV in humans. The first case report describes a 49-year old male PV patient with genital blisters. The patient was refractive to therapy with prednisone, dapsone, methotrexate and gold sodium thiomalate. Due to this immunosuppressive therapy the patient developed a Kaposi sarcoma. Therefore methotrexate was switched to systemic sirolimus, which resulted in an improvement of the sarcoma and a fast and complete remission of the PV. The second case report describes a 49-year old patient with mcPV, with refractive skin lesions despite therapy with prednisone, azathioprine and intravenous immunoglobulins. Azathioprine was stopped, the dose of prednisone was reduced, and systemic therapy with sirolimus was started. This resulted in complete remission after 2 weeks. The quick response displayed in these two case reports, and the effective intradermal treatment in the PV mouse model, have been used to argue that sirolimus exerts its protective effect by directly acting on keratinocytes, as opposed to acting as a systemic immunosuppressive agent that inhibits autoantibody formation. Therefore, in chapter 7 we evaluated whether topical sirolimus could be used as therapy for three PV patients.
References
1. Introduction


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