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Development of Specific Immunoadsorbers for the Treatment of Human Pemphigus Diseases

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Lübeck, 19/03/2012 _____

Jana Langenhan

Development of Specific Immunoadsorbers for the Treatment of Pemphigus Diseases

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Contents

1. Summary

Pemphigus diseases are a class of rare intraepidermal blistering disorders. Most frequent are pemphigus vulgaris and pemphigus foliaceus. Both are characterised by the presence of autoantibodies directed against calcium dependent adherins (cadherins), namely desmoglein 1 and desmoglein 3. These autoantibodies are pathogenic and cause acantholysis by interfering with the cell-to-cell contact of keratinocytes. In addition to systemic immunosuppression, pemphigus can be treated with plasmapheresis. Thereby, either plasma exchange or removal of total IgG is carried out at present. To minimise side effects, it would be advantageous to use adsorption columns that exclusively eliminate anti-desmoglein antibodies. This thesis was devoted to the generation of specific adsorber matrices for this purpose.

The extracellular domains of desmoglein 1 and 3 were expressed in HEK 293T cells and purified via immobilised metal ion affinity chromatography. Suitable solid supports were sought for immobilisation of these antigens. Out of six matrices tested, NHS-activated Sepharose and aldehyde agarose were most efficient in binding anti-Desmoglein antibodies, as was determined by detection of specific IgG in the flow through of the columns. The amount of anti-desmoglein antibodies in spiked sera could be decreased to 20 % in the mean. After optimisation, 200 µL serum could be processed with 20 µL desmoglein matrix. Adsorption was specific, since the level of IgG directed against antigens not related to pemphigus was not influenced. *In vitro* assays using human keratinocytes showed significantly decreased pathogenicity of adsorbed sera.

It is intended to reuse desmoglein adsorbers for one patient. Acidic regeneration of the matrices was possible, but adsorption capacity dropped in the course of this process by 35 % in case of desmoglein 1 matrices and by 12 % in case of desmoglein 3 matrices. To enhance stability and focus on relevant epitopes, several recombinant desmoglein mutants lacking putatively irrelevant extracellular domains were created. Only one desmoglein 1 variant, consisting of the extracellular domains 1 and 5, was found to react with anti-desmoglein 1 IgG from patients' sera. But this protein was less efficient than the full length extracellular domain of desmoglein 1 in removing pathogenicity of spiked sera. There was no such desmoglein 3 mutant capable of binding autoantibodies. Since most anti-desmoglein 3 antibodies belong to the IgG4 subclass, removal of total IgG4 by a specific antibody was conducted. However, desmoglein 3 matrices were more powerful in abolishing pathogenic effects of pemphigus sera. Both desmoglein 1 and desmoglein 3 were highly efficient in adsorbing autoantibodies from a series of individual pemphigus sera. The amount of anti-desmoglein 1 IgG was decreased to 1-40 % of the initial level, anti-desmoglein 3 IgG to < 20 % after the first run.

Use of desmoglein adsorbers in pemphigus therapy will possibly improve treatment of pemphigus and rapidly alleviate clinical symptoms.

2. Introduction

2.1 Pemphigus Diseases

Pemphigus diseases are a class of very rare autoimmune skin disorders. Incidence ranges from 0.8 to 16 new cases per million inhabitants per year[88]. In some population groups of the Middle East (Jewish, Mediterraneans, Northern Indians, Persians), incidence is higher[102]. Four different types of pemphigus exist: pemphigus foliaceus (PF), pemphigus vulgaris (PV), paraneoplastic pemphigus (PNP) and IgA pemphigus[3,80,106,115]. Most common is PV, followed by PF[88,102]. There is also an endemic form of PF, called *fogo selvagem*, which occurs in some areas in South America[37,44].

Pemphigus diseases are potentially lethal if left untreated[11]. The clinical picture is characterised by intraepidermal blister formation. These blisters are usually flaccid and rupture easily, leaving crusts and erosions. Patients suffering from PF have scaly, crusty skin lesions distributed along the trunk, face and scalp[3,130]. By histological analysis of skin biopsies, loss of cell-cell adhesion between keratinocytes (acantholysis) is observed (Figure 1). Epidermal splitting occurs between granular layers and is accompanied by inflammatory infiltrates[80,130]. PV patients show lesions and erosions on mucous membranes. Usually the mouth is affected first, but throat, nasal mucosae, conjunctiva, anus and genital mucosae may also be involved[3,106,130]. About 50 % of PV patients have additional blistering on the skin similar to PF[60]. In these cases, epidermal cleavage is observed right above the basal layer by histology[80,115,130] (Figure 1).

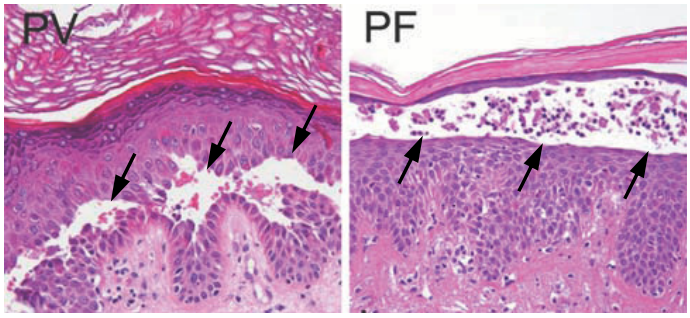

Figure 1: Typical histology from epidermal lesions of pemphigus patients
Derived from Waschke, 2008[130]. Hematoxylin eosin-stained paraffin section from a PV patient (left) shows suprabasal epidermal cleavage. In the section from a PF patient (right), superficial granular blistering can be observed. Arrows indicate sites of cleavage.

In 1964, the presence of autoantibodies in pemphigus patients was demonstrated for the first time[21]. In the following years, desmosomes were identified to be the target structures for these antibodies[66,67]. Desmosomes are multiprotein cell adhesion structures, which are most numerous in tissues subjected to mechanical stress like epithelia and the myocardium[100,130]. Their composition varies between tissues and between differentiation states of cells in the same tissue[67]. They consist of desmogleins, desmocollins, plakoglobin and

plakophillin and are linked to the cytoskeleton via desmoplakin[130] (Figure 2). Further analyses of pemphigus antibodies revealed desmogleins as main autoantigens: desmoglein 1 (Dsg1) in case of PF and desmoglein 3 (Dsg3) in case of PV[9,45,54]. PV patients with epidermal lesions usually have additional autoantibodies against Dsg1[7,41,46].

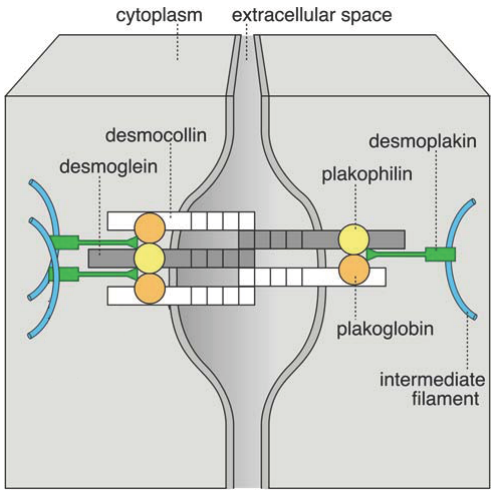

Figure 2: Schematic composition of desmosomes
Modified from Waschke, 2008[130]. Desmogleins and desmocollins are transmembrane proteins. They interact with each other via their extracellular domains. The cytoplasmic parts of both proteins bind to plakophillin, plakoglobin and desmoplakin. Desmoplakin forms the linkage to the intermediate filament.

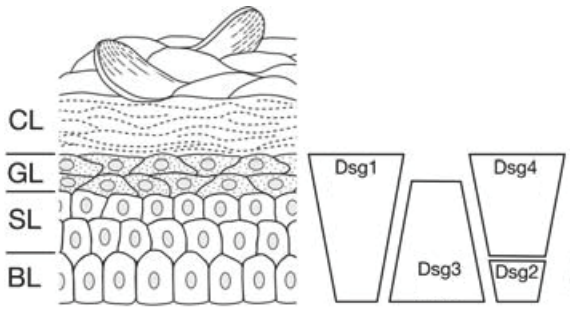

Figure 3: Expression patterns of desmogleins in the epidermis
Modified from Waschke, 2008[130]. The schematic drawing of the epidermis (left) indicates basal (BL), spinous (SL), granular (GL) and corneal (CL) layer. On the right, the expression patterns of Dsg isoforms are illustrated.

Desmogleins belong to the cadherin protein family[9,134] and are necessary for desmosome formation[53]. Four different isoforms of desmogleins exist (Dsg1-4), which are expressed differently with respect to the tissue: Dsg1 is present predominantly in stratified corneal epithelium (superficial epidermis). Dsg2 is expressed in simple epithelia and the urothelium. Expression of Dsg3 is confined to stratified epithelia (lower epidermis), including mucosae. The tissue distribution of Dsg4 includes skin (hair follicles) and several simple epithelia[3,130]. A schematic illustration of the location of Dsg isoforms in the skin is shown in Figure 3.

Desmogleins are glycosylated transmembrane proteins with their C-terminus being located intracellularly (Figure 4). Similar to classical cadherins, such as E-cadherin or C-cadherin, the extracellular part of Dsg1 and Dsg3 consists of five domains, termed EC1-5,

four of which are so-called cadherin domains (EC1-4)[9]. EC1 is the most N-terminal domain and harbours a signal sequence and a propeptide, which are cleaved during the maturing process of the proteins[95,100]. The membrane-proximal extracellular domain is usually referred to as EC5, although its similarity to cadherin repeats is limited[90].

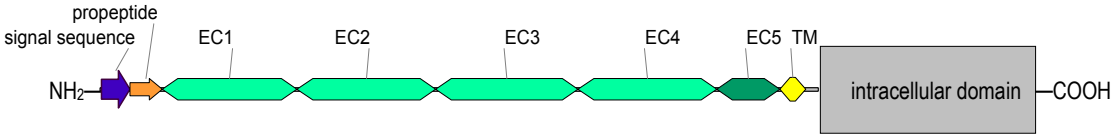

Figure 4: Schematic structure of Dsg1 and Dsg3
Structure of Dsg1 and Dsg3 from N-terminus (left) to C-terminus (right): signal sequence, propeptide, extracellular domains (EC) 1-5, transmembrane domain (TM) and intracellular domain.

The three dimensional structure of Dsg1 and Dsg3 heavily depends on the presence of Ca^{2+} ions[45,46], which also play a key role in the formation of desmosomes[99,133]. Every cadherin domain contains a highly conserved calcium-binding motif[55,90]. In addition, cadherin-cadherin interactions depend on the concentration of Ca^{2+}[101].

Adhesive strength of desmogleins is most likely mediated by the outermost N-terminal part of EC1 through a highly conserved tryptophan residue present in both classical and desmosomal cadherins[55,94]. By fitting into a hydrophobic pocket on an opposing molecule (e.g. on an adjacent cell), cadherins form head-to-head (*trans*) homodimers. In addition, *cis*-dimers consisting of two neighbouring cadherins have been observed[25,101,125,137]. This is likely to be the case for desmogleins, too, because the three-dimensional structure is conserved between the different cadherin families[90]. There are also models derived from *in vitro* studies proposing extensive interdigitation of cadherin molecules[32,55,94]. The *in vivo* situation is still under investigation, but seems to be in-between the findings gained from the different *in vitro* systems[55]. Interestingly, epitopes of anti-Dsg autoantibodies map predominantly to the N-terminal part of both antigens[3,31,47,73,92,111], which is considered to be most important for cell adhesion according to these models.

2.2 Blister Induction by anti-Dsg Autoantibodies

Pemphigus sera and purified IgG have been shown to cause acantholysis in passive transfer mouse models[5,6,8,41], skin organ cultures[89,105] and *in vitro* assays using keratinocytes[61,66,131]. Although the pathogenic relevance of anti-Dsg antibodies has been a matter of debate[4,49,89,121], today it is generally accepted that human anti-Dsg antibodies alone are sufficient to induce disease. This is based upon the following major findings:

I. In individual patients, serum levels of anti-Dsg IgG correlate well with disease activity[60].
II. Blister location is consistent with the expression patterns of Dsg1 and Dsg3 in the different tissues[76,114].

III. Purified anti-Dsg IgG induce blisters by passive transfer into neonatal mice, whereas patients' sera depleted of anti-Dsg antibodies do not[5,6,111].

IV. During pregnancy, anti-Dsg IgG can cross the placenta, causing neonatal pemphigus of the foetus, which spontaneously subsides some weeks after birth[14,117].

The mechanisms of acantholysis and blister formation by autoantibodies are still controversially discussed. Activation of complement apparently is not required[117,130], although C3 deposition can be observed in skin biopsies of pemphigus patients[80,102,117]. In fact, Fab fragments, $F(ab')_2$ fragments or single-chain variable-region fragments (scFvs) directed against desmogleins can cause blisters when passively transferred into neonatal mice, although they are not able to fix complement[44,62,79,97]. There are some indications supporting the assumption that anti-Dsg IgG mediate pathogenic effects by steric hindrance of Dsg-Dsg interactions[57,58,126]. However, this does not seem to be the sole cause for acantholysis, since clustering[29,93] and degradation[12,29,34,35,64] of desmogleins has been observed in keratinocytes upon incubation with pathogenic anti-Dsg antibodies. Also, keratinocytes incubated with PV IgG on ice do not show reduced adhesive strength. This argues for the involvement of metabolic events in acantholysis, because enzymatic activities are inhibited near 0 °C[29]. Indeed, numerous signalling events have been observed in pemphigus pathogenesis including retraction of actin and keratin filaments[19,28,48,82,132], relocalisation of plakoglobin[28] and desmoplakin[29], phosphorylation of p38 mitogen-activating protein kinase (p38 MAPK) and heat shock protein (HSP) 27[17-20,65,72], inhibition of Rho A[118,120,132], activation of c-Src kinase and epidermal growth factor receptor kinase[33], Dsg3 internalisation via tyrosine kinases[38] and activation of several apoptotic pathways[16,72,98]. Apoptosis, however, seems to be a consequence rather than the cause of acantholysis[16,72,108]. In addition, desmosomes are highly dynamic structures[16,70] and there is accumulating evidence that anti-Dsg antibodies do not destroy existing desmosomes, but hamper the formation of new desmosomes[12,34,64,77,83,93].

As mentioned above, conformation of both classical and desmosomal cadherins only can be maintained by the binding of calcium ions. Therefore, it is not surprising that antibody binding to desmogleins, too, depends on the concentration of Ca^{2+}[7,45,46,126].

Overall, the effects of anti-Dsg3 antibodies have been more extensively studied than the effects of anti-Dsg1 antibodies. Interestingly, not all autoantibodies directed against these two proteins are pathogenic[22,62,68,77,97,126,135]. Antibodies recognising epitopes on the N-terminus of desmogleins appear to be more pathogenic than those binding to epitopes residing on EC4 or EC5[8,84,136], although the sites of antibody binding on the extracellular domains is preserved during different activity states of PF and PV[31,92]. Intracellular epitopes are rare and appear only at later stages of disease. The reason for this is speculated to

be intramolecular epitope spreading[124]. These epitopes do not contribute to pathogenicity. In addition to the site of antibody binding, the nature of recognised epitopes seems to be important for acantholysis. Almost all pathogenic anti-Dsg antibodies characterised so far bind to conformational epitopes, whereas the recognition of linear epitopes is less frequently associated with pathogenicity[8,42,52,97,111,126].

2.3 Therapy

Despite the well established causal connection of anti-Dsg autoantibodies with acantholysis and blister formation, therapy of pemphigus is based upon systemic immunosuppression using high-dose corticosteroids, various immunosuppressive and anti-inflammatory agents, intravenous immunoglobulins and cytotoxic substances[24,63,102,130]. This is accompanied with side effects that are partially severe and do not only decrease life quality of the patients, but are the primary cause of morbidity and mortality in these diseases[37,75,102]. They include systemic infections, diabetes mellitus, osteoporosis and thromboses[109]. In severe cases, in which pemphigus is therapy refractory or even resistant, immunoadsorption may be applied as adjuvant therapy[43,113,140] (Figure 5). During immunoadsorption, also called immune apheresis, the plasma of a patient is processed with an affinity column binding immunoglobulins, predominantly IgG[91,127]. Although there is a lack of controlled, randomised trials, immunoadsorption shows high therapeutic efficiency[43,109,139]. Even so, the immunoadsorption columns available at present all have one major drawback: They do not only bind disease-associated antibodies, but all IgG and to a certain extent IgM, IgA and IgE. This results in removal of immunoglobulins necessary for the defence against pathogens in addition to autoantibodies, which makes patients more susceptible to infections.

The application of total IgG adsorbers for the treatment of pemphigus is the more intriguing if considering that it was shown already in the mid 1990s that removal of anti-Dsg antibodies via recombinant desmogleins is sufficient to abolish the pathogenicity of patients' sera[5,6]. Nevertheless, no specific adsorption devices for use on humans have been developed so far. Building the basis for such adsorption columns is the main issue of this work.

Apart from adsorption on desmoglein matrices, other approaches to specifically impair the effects of anti-Dsg antibodies on desmosomes have been pursued. Anti-idiotypic antibodies have been shown to abolish pathogenic effects of PF sera in neonatal mice and have been suggested as possible ligands of immunoadsorption columns[2]. The research group immunised rabbits with anti-Dsg IgG to produce anti-idiotypic antibodies. But reactivity of these antibodies with PF sera was diverse, indicating that the epitope profile of PF patients varies. To cover all anti-Dsg antibodies, it would probably be necessary to use a diversity

of anti-idiotypic antibodies. Obtaining this diversity and achieving constant quality by immunisation of animals will be difficult.

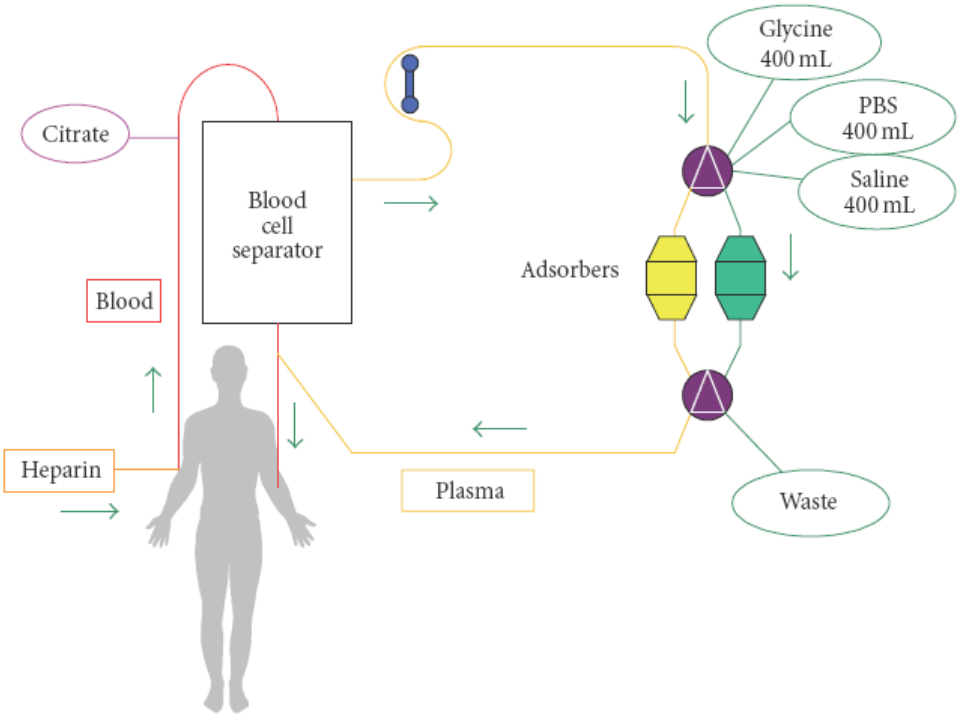

Figure 5: Schematic diagram of immunoadsorption
Derived from Blaha, 2010[23]. Blood is drawn from the patient and supplemented with heparin and citrate to prevent coagulation. Then the plasma is separated from the cells and flows over one of two adsorption columns. After adsorption, plasma is mixed with the cells and runs back into the patient. While one column is used for plasma processing, the other column is regenerated with glycine and PBS and subsequently washed with saline. The plasma flow switches from the first to the second column as soon as regeneration is finished.

Some years ago, a peptide has been identified that is able to bind both anti-Dsg1 and anti-Dsg3 antibodies[73]. This peptide caused clinical improvement of skin lesions of one patient with mucocutaneous PV upon topical administration[10]. Topical application does not prevent the formation of lesions, but aids their healing. In addition, the development of topicals for administration on mucosae, which are predominantly affected in PV, is difficult and restricted. This limits the use of this peptide. Also, diversity of epitope recognition in individual patients might prevent general success of an agent which covers only such a small fraction of desmogleins. Similar restrictions apply to a tandem peptide that was developed to stabilise Dsg-Dsg interactions by binding to their N-termini[57].

p38 MAPK has also been proposed to be a novel therapeutic target. In mice, inhibition of phosphorylation of p38 MAPK prevented blister formation by PV IgG[20]. However, p38 MAPK is involved in numerous signalling pathways and blocking this kinase might come along with severe side effects.

Another possible therapeutic target in PV is caspase 3. This protease is essential for Fas mediated apoptosis. Although apoptosis has been shown to be a downstream event in pemphigus blister formation[72], blocking caspase 3 in mice prevented acantholysis upon injection of PV antibodies[96]. This effect was observed when mice were pretreated with a cas-

pase 3 inhibitor. Pretreatment of human pemphigus patients, however, is no therapeutic option, because usually the patients already suffer from blisters when they call on a physician. Prevention of flares in ongoing disease is not possible, also, because flares cannot be predicted. Since apoptosis is an important means of the body to prevent tumour formation or viral infections, circumventing this mechanism in general by permanently inhibiting caspase 3 might lead to unwanted side effects.

Taken together, specific adsorption of anti-Dsg antibodies seems to be the most promising and safest new therapeutic option for the treatment of PV and PF. In addition, immunoadsorption treatment has the advantage of a very fast improvement of the clinical picture of pemphigus patients[43,113]. This study aimed for the development of immunoadsorption matrices based on recombinant desmogleins to exclusively remove the pathogenic agents from human pemphigus sera.

3. Materials and Methods

3.1 Chemicals, Buffers and Media

1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)	GE Healthcare Europe, Germany
acetic acid	VWR International, Germany
albumin/azide	EUROIMMUN, Germany
aldehyde blocking buffer	1 M Tris 5 mM $CaCl_2$ pH = 7.4
aldehyde coupling buffer	10-50 mM HEPES 0.5 M NaCl 5 mM $CaCl_2$ pH = 7.2
aldehyde washing solution	1 M NaCl 5 mM $CaCl_2$
AminoLink Plus Coupling Resin	Fisher Scientific, Germany
amphotericin B (100 x stock solution)	PAA Laboratories, Germany
anti-human IgG POD-conjugate for ELISA (ready-to-use)	EUROIMMUN, Germany
calcium chloride dihydrate	Hassa, Germany
carboxy coupling buffer	0.5 M NaCl 0.1 M NaAc 5 mM $CaCl_2$ pH = 4.5
carboxy washing solution	1 M NaCl 5 mM $CaCl_2$ pH = 4.5-5
casein blocking buffer	EUROIMMUN, Germany
casein sample buffer	EUROIMMUN, Germany
chloramphenicol	Roth, Germany
Coomassie brilliant blue G250	Th. Geyer Hamburg, Germany
Coomassie destaining solution	40 % methanol 10 % acetic acid
covering medium	EUROIMMUN, Germany
Dispase in Hank's Balanced Salt Solution (5 mg/mL)	STEMCELL Technologies SARL, France
DMEM High Glucose, supplemented with L-glutamine and sodium pyruvate	PAA Laboratories, Germany
Dsg-TBS	20 mM Tris 150 mM NaCl 5 mM $CaCl_2$ pH = 7.4

Dsg-TBS/20 % glycerol	20 mM Tris 150 mM NaCl 5 mM $CaCl_2$ 20 % glycerol pH = 7.4
ELISA stopping solution	EUROIMMUN, Germany
ELISA substrate	EUROIMMUN, Germany
ELISA washing buffer (10 x stock solution)	EUROIMMUN, Germany
elution buffer	0.1 M glycine 5 mM $CaCl_2$ 0.2 % Tween® 20 pH = 2.5
ethanolamine hydrochloride	Sigma-Aldrich Chemie, Germany
ExGen 500 *in vitro* Transfection Reagent	Fermentas Molecular Biology, Germany
FBS (Mycoplex)	PAA Laboratories, Germany
formaldehyde	Sigma-Aldrich Chemie, Germany
glycerol	Gerbu Biotechnik, Germany
glycine	Roth, Germany
HEPES (PUFFERAN®)	Roth, Germany
hydrochloric acid	Th.Geyer, Germany
imidazole	Sigma-Aldrich Chemie, Germany
Keratinocyte Growth Medium 2 (KGM2) Kit	PromoCell, Germany
LB agar (ready-to-use mixture)	Roth, Germany
LB medium (ready-to-use mixture)	Roth, Germany
magnesium chloride hexahydrate	Gerbu Biotechnik, Germany
methanol	VWR International, Germany
methyl violet 2B	Sigma-Aldrich Chemie, Germany
neutralisation buffer	1 M Tris 5 mM $CaCl_2$ pH = 7.6
NHS blocking buffer 1	0.5 M ethanolamine 0.5 M NaCl 5 mM $CaCl_2$ pH = 8.3
NHS blocking buffer 2	0.5 M ethanolamine 0.5 M NaCl pH = 8.3
NHS coupling buffer 1	10 mM HEPES 0.5 M NaCl 5 mM $CaCl_2$ pH = 8.0

NHS coupling buffer 2	0.2 M Na_2CO_3 0.5 M NaCl pH = 8.3
NHS washing buffer 1	0.1 M sodium acetate 0.5 M NaCl 5 mM $CaCl_2$ pH = 4.0
NHS washing buffer 2	0.1 M sodium acetate 0.5 M NaCl pH = 4.0
NHS-activated Sepharose™ 4 Fast Flow	VWR International, Germany
N-hydroxysuccinimide (NHS)	GE Healthcare Europe, Germany
NuPAGE® MES-SDS running buffer (20 x stock solution)	Invitrogen, Germany
NuPAGE® Transfer Buffer (20 x stock solution)	Invitrogen, Germany
PBS (ready-to-use mixture)	EUROIMMUN, Germany
PBS coating buffer	EUROIMMUN, Germany
PBS/T	0.2 % Tween® 20 in PBS
PBS/U	1 x PBS 8 M urea pH = 7.4
penicillin/streptomycin (100 x stock solution)	PAA Laboratories, Germany
Ponceau S partical	Sigma-Aldrich Chemie, Germany
sodium acetate	Sigma-Aldrich Chemie, Germany
sodium acide (9 % stock solution)	EUROIMMUN, Germany
sodium ampicillin	Gerbu Biotechnik, Germany
sodium chloride	Th.Geyer, Germany
sodium cyanoborohydride	Sigma-Aldrich Chemie, Germany
sodium hydrogen carbonate	VWR International, Germany
sodium hydroxide (5 M solution)	Th.Geyer, Germany
Spectra® Multicolor Broad Range Protein Ladder	Fermentas Molecular Biology, Germany
Sterogene IMAC Ni-resin	Biotech-IgG, Denmark
Taubert buffer (10 x stock solution)	EUROIMMUN, Germany
TNIMCa (10 x stock solution)	55 mM Tris 1.8 M NaCl 220 mM imidazole 550 mM $MgCl_2$ 55 mM $CaCl_2$ pH = 8.0

Toyopearl AF-Carboxy-650	Tosoh Bioscience, Germany
Toyopearl AF-Formyl-650	Tosoh Bioscience, Germany
Toyopearl AF-Tresyl-650	Tosoh Bioscience, Germany
Tresyl blocking buffer	0.1 M Tris 0.5 M NaCl 5 mM $CaCl_2$ pH = 8.5
Tresyl coupling buffer	see NHS coupling buffer
Tris-b	Gerbu Biotechnik, Germany
Tween® 20	Gerbu Biotechnik, Germany
UltraLink Biosupport	Fisher Scientific, Germany
urea	Gerbu Biotechnik, Germany
Westernblot blocking buffer (10 x stock solution)	EUROIMMUN, Germany
Westernblot substrate	EUROIMMUN, Germany
Westernblot washing buffer (10 x stock solution)	EUROIMMUN, Germany
phalloidin-TRITC	Sigma-Aldrich Chemie, Germany

3.2 Sera and Antibodies

Pemphigus sera and antibodies as well as control sera were collected by the Institute of Dermatology and Venerology, University of Lübeck. All pemphigus patients were under medical treatment and had positive ELISA results with respect to autoantibodies against Dsg1 and/or Dsg3.

Table 1: Antibodies and conjugates

antibody	purchased from
anti-Dsg3 mouse monoclonal antibody	santa cruz biotechnology, Germany
anti-His-tag mouse monoclonal antibody	Merck Chemicals, Germany
anti-p38 MAPK rabbit polyclonal antibody	New England Biolabs, Germany
anti-P-p38 MAPK rabbit monoclonal antibody	New England Biolabs, Germany
goat anti-human IgG, $F(ab')_2$-HRP	Dianova, Germany
goat anti-rabbit IgG-HRP	Sigma Aldrich Chemie, Germany
monoclonal anti-human IgG4, Fc-fragment specific	Biomol, Germany
mouse anti-human IgG4 (Fc)-HRP	BIOZOL Diagnostica Vertrieb, Germany
polyclonal goat anti-human IgG (H+L)-Cy2	Dianova, Germany
polyclonal goat anti-human IgG, Fc-fragment specific	Dianova, Germany
polyclonal goat anti-mouse IgG (H+L)-Cy2	Dianova, Germany
polyclonal goat anti-mouse IgG-AP	Dako Deutschland, Germany

Since Pemphigus is a very rare disease and serum samples are difficult to obtain, artificial sera pools were created for use in experiments with high serum consumption. Therefore, antibodies from four PF- and PV-patients each, which were obtained during immunoadsorption therapies with TheraSorb™ (Miltenyi Biotec, Germany), were diluted in sera of healthy blood donors. This way, large amounts of artificial pemphigus sera could be

created. The resulting ELISA titre of the PF-pool was 471 RU/mL against Dsg1 and of the PV-pool 400 RU/mL against Dsg3.

Rabbit sera against Dsg1 and Dsg3 were purchased from Eurogentec Deutschland (Germany). Dsg1(ec)-His and Dsg3(ec)-His were used for the immunisation.

For detailed information on purified antibodies and conjugates see Table 1.

3.3 Cloning

3.3.1 List of Dsg Expression Plasmids

All expression plasmids were derived from pTriEx-1.1 (Merck Biosciences, Germany). The respective expression plasmids for Dsg1(ec)-His, Dsg3(ec)-His, Dsg1(ec)-TM and Dsg3(ec)-TM were already available at EUROIMMUN[107]. A list of self cloned vectors is shown in Table 2.

Table 2: List of expression plasmids

plasmid name	expressed protein
pTriEx-1 TM_Dsg1	none/helper construct
pTriEx-1 TM_Dsg3	none/helper construct
pTriEx-1 pre_pro_Dsg1_EC1-4_TM	Dsg1_EC1-4_TM
pTriEx-1 pre_pro_Dsg1_EC1-3_TM	Dsg1_EC1-3_TM
pTriEx-1 pre_pro_Dsg1_EC1-2_TM	Dsg1_EC1-2_TM
pTriEx-1 pre_pro_Dsg1_EC1_TM	Dsg1_EC1_TM
pTriEx-1 pre_pro_Dsg1_EC1+3_TM	Dsg1_EC1+3_TM
pTriEx-1 pre_pro_Dsg1_EC1+4_TM	Dsg1_EC1+4_TM
pTriEx-1 pre_pro_Dsg1_EC1+5_TM	Dsg1_EC1+5_TM
pTriEx-1 pre_pro_Dsg3_EC1-4_TM	Dsg3_EC1-4_TM
pTriEx-1 pre_pro_Dsg3_EC1-3_TM	Dsg3_EC1-3_TM
pTriEx-1 pre_pro_Dsg3_EC1-2_TM	Dsg3_EC1-2_TM
pTriEx-1 pre_pro_Dsg3_EC1_TM	Dsg3_EC1_TM
pTriEx-1 pre_pro_Dsg3_EC1+3_TM	Dsg3_EC1+3_TM
pTriEx-1 pre_pro_Dsg3_EC1+4_TM	Dsg3_EC1+4_TM
pTriEx-1 pre_pro_Dsg3_EC1+5_TM	Dsg3_EC1+5_TM
pTriEx-1 pre_pro_Dsg1_EC1+2+5_TM	Dsg1_EC1+2+5_TM
pTriEx-1 pre_pro_Dsg3_EC1+Dsg1_EC5_TM	Dsg3_EC1+Dsg1_EC5_TM
pTriEx-1 pre_pro_Dsg1_EC1+5_His	Dsg1_EC1+5_His

All Dsg variants were expressed with the authentic signalling sequence (“pre”) and propeptide (“pro”). Both sequences were cleaved during the maturing process of the proteins. In case of Dsg1, EC1 is represented by aa 50-158, EC2 by aa 159-270, EC3 by aa 271-385, EC4 by aa 386-497, EC5 by aa 498-548 and TM by aa 549-569. Concerning Dsg3, EC1 consists of aa 49-157, EC2 of aa 158-267, EC3 of aa 268-384, EC4 of aa 385-498, EC5 of aa 499-614 and TM of aa 617-641.

Including glycosylation, both Dsg1(ec)-His and Dsg3(ec)-His have an apparent molecular weight of approximately 80 kDa after cleavage of signal sequence and propeptide, as was determined by Westernblot analysis. Dsg1_EC1+5_His has a size of 50 kDa under the same conditions.

3.3.2 Construction of Dsg Expression Plasmids

Vector NTI Advance 11 (Invitrogen, Germany) was used for the planning of all cloning procedures. This includes restriction analyses, generation of primers and creation of virtual agarose gel pictures for analytical PCR and control restriction.

Table 3: List of primers

primer	sequence	restriction enzymes
sense TM_Dsg1	GATCCGCTGGCATTGGACTCCTCATCATGGGATTCTT GGTCTTAGGATTGGTCCCATTTTTGATGATCC	*Bam*HI/*Xho*I
asense TM_Dsg1	TCGAGGATCATCAAAAATGGGACCAATCCTAAGACCA AGAATCCCATGATGAGGAGTCCAATGCCAGCG	*Bam*HI/*Xho*I
sense TM_Dsg3	ATACTCGAGCTGGGGCCTGCCGCCATCGGCCT	*Xho*I
asense TM_Dsg3	ATATGTCGACGGTCAACAGCAGAAGGGGGG	*Sal*I
sense pre_Dsg1	ATACCATGGACTGGAGTTTCTTCAGAGTAG	*Nco*I
asense Dsg1_EC5	TTATGGATCCTCCAGGACCAAAATGTACATTGT	*Bam*HI
asense Dsg1_EC4	TTATGGATCCTCCGTTCGGCTCTGTATTAGTC	*Bam*HI
asense Dsg1_EC3	TTATGGATCCTCCAAACACTGGGCCTTCAATT	*Bam*HI
asense Dsg1_EC2	TTATGGATCCTCCTTCCATGTAAGGGATATTATCATT GACATC	*Bam*HI
asense Dsg1_EC1	TTATGGATCCTCCTGAAAACACTGGAGGGTTG	*Bam*HI
asense Dsg1_EC1-*Kpn*I	TTATGGTACCTGAAAACACTGGAGGGTTG	*Kpn*I
sense Dsg1_EC3	TATTGGTACCCAGTCTTCATATACCATAGA	*Kpn*I
sense Dsg1_EC4	TATTGGTACCCGTCCAGGTTCAAAGACAT	*Kpn*I
sense Dsg1_EC5	TATTGGTACCACTAAAATTACTACCAATACT	*Kpn*I
sense pre_Dsg3	ATACCATGGGGCTCTTCCCCAGAACTACAGGGGCTC	*Nco*I
asense Dsg3_EC5	ATACTCGAGCCTCCCTGAGTGCGGCCTGCCATACCTG	*Xho*I
asense Dsg3_EC4	TATTCTCGAGTCCTCCTTTTTCGAGGACAGCTG	*Xho*I
asense Dsg3_EC3	TATTCTCGAGTCCTCCAGGACGGAATGCAATTCC	*Xho*I
asense Dsg3_EC2	TATTCTCGAGTCCTCCTCTAAACATTGGGAAGTTATC	*Xho*I
asense Dsg3_EC1	TATTCTCGAGTCCTCCTGAAAATACTGGAGGATTATC	*Xho*I
asense Dsg3_EC1-*Bam*HI	TATTGGATCCTGAAAATACTGGAGGATTATC	*Bam*HI
sense Dsg3_EC3	TTATGGATCCGACTCTCAGTATTCAGCAC	*Bam*HI
sense Dsg3_EC4	TTATGGATCCGCTTCCAAGACATTTACTGT	*Bam*HI
sense Dsg3_EC5-*Bam*HI	TTATGGATCCGATGCAGTTTGCAGTTCTT	*Bam*HI
asense Dsg1_EC2-*Lgu*I	ATGCTCTTCCAGTTTCCATGTAAGGGATATTAT	*Lgu*I
sense Dsg1_EC5-*Lgu*I	ATGCTCTTCCACTAAAATTACTACCAATACTGG	*Lgu*I
asense Dsg1_EC5-*Sal*I	ATAGTCGACAGGACCAAAATGTACATTGTCTGATAAC AAATCTTTGGCTC	*Sal*I
asense Dsg3_EC1-*Kpn*I	GCGGTACCTGAAAATACTGGAGGATTAT	*Kpn*I

Table 3 lists the used primers. For the cloning of the membrane-bound Dsg variants, vectors containing the transmembrane domain of Dsg1 and Dsg3 were made. TM_Dsg1 was synthesised as a linker and inserted into pTriEx-1.1 via BamHI and XhoI. TM_Dsg3 was used as PCR-product and inserted via XhoI and SalI. This way, Dsg1 fragments could be introduced with NcoI and BamHI whereas Dsg3 fragments were inserted via NcoI and XhoI. Connection between different extracellular domains of Dsg1 was achieved with KpnI or LguI, in case of Dsg3 with BamHI. To remove the transmembrane domain from Dsg1_EC1+5_TM, pTriEx-1.1 was cut with NcoI and XhoI before Dsg1_EC1+5 was inserted as PCR product made of pTriEx-1.1 pre_pro_Dsg1_EC1+5_TM and the appropriate primers (sense pre-Dsg1 and asense Dsg1_EC5-*Sal*I). Fusion of Dsg1_EC5 to Dsg3_EC1

was achieved with KpnI, insertion into pTriEx-1.1 TM_Dsg1 was done via NcoI and BamHI.

3.3.3 Amplification of DNA Fragments by PCR

For cloning purposes, enzymes and buffers of the High Fidelity PCR Enzyme Mix (Fermentas Molecular Biology, Germany) were used. Taq DNA Polymerase LC (Fermentas Molecular Biology, Germany) was used for analytical PCR. Composition of the reaction mix was as follows:

High Fidelity reaction mix

1 x High Fidelity PCR Buffer
0.2 mM dNTPs each
1 µM primers each
10 ng template DNA
2.5 U High Fidelity PCR Enzyme Mix

Taq DNA Polymerase LC reaction mix

1 x PCR Buffer
2 mM $MgCl_2$
0.2 mM dNTPs each
1 µM primers each
10 ng template DNA or 2 µL bacterial culture
1.25 U Taq Polymerase LC

PCR procedure

melting: 30 s at 94 °C
annealing: 45 s at 56 °C
amplification: 1 min per kb at 72 °C
repetition: 30 times

PCR cleanup was done with NucleoSpin® Extract II (Macherey-Nagel, Germany).

3.3.4 Digestion by Endonucleases

All restriction enzymes and appropriate buffers were purchased from Fermentas Molecular Biology (Germany). Both conventional and Fast Digest® enzymes were used. Composition of the restriction mix varied with respect to the different restriction enzymes and was arranged according to the manufacturer's instructions.

Purification of digested DNA fragments was done either directly with NucleoSpin® Extract II (Macherey-Nagel, Germany) or by agarose gel preparation using the same kit.

3.3.5 Ligation and Transformation

For ligation of DNA fragments, the Rapid DNA Ligation Kit of Fermentas Molecular Biology (Germany) was used. The reaction was done in 10 min at room temperature (RT) in a total volume of 20 µL consisting of 1 µL vector DNA, 3-5 µL insert DNA, 1 x Rapid Ligation Buffer and 5 Units T4 DNA ligase.

After ligation, the reaction mix was directly used for transformation into heat competent *E. coli* (XL-2). DNA was added to the cell suspension and incubated for 10 min on ice. The heat shock was done at 42 °C for 45 s, followed by a one-minute-incubation on ice. Then four volumes of LB medium were added and the cells were incubated at 37 °C for 30 min while shaking. After centrifuging for 2-3 min at 4 °C and full speed, most of the supernatant was removed. The cells were resuspended in the remaining supernatant and plated onto LB agar plates supplemented with chloramphenicol (34 µg/mL) and ampicillin (100 µg/mL) for selection. Plates were stored over night at 37 °C.

3.3.6 Plasmid Preparation and Sequencing

Bacterial clones from agar plates were tested by analytical PCR. Correct ones were then inoculated in 5 mL LB medium with 34 µg/mL chloramphenicol and 100 µg/mL ampicillin and incubated over night at 37 °C under shaking. On day two plasmids were purified using NucleoSpin® Plasmid (Macherey-Nagel, Germany) and sequenced at Eurofins MWG Operon (Germany). Evaluation of DNA sequences was done with ContigExpress 11 and Vector NTI Advance 11 (Invitrogen, Germany).

3.3.7 Large Scale Plasmid Purification

For preparation of larger amounts of plasmid DNA, NucleoBond® Xtra Midi or PC 10,000 (Macherey-Nagel, Germany) was utilised according to the manufacturer's instructions. The concentration of the purified DNA was set to 0.5 µg/µL.

3.4 Immunofluorescence Studies on Dsg Variants

3.4.1 Transfection of HEK 293T Cells

HEK 293T cells were seeded either onto BD Falcon™ CultureSlides or onto glass slides (EUROIMMUN, Germany) with a density of $3.75 \cdot 10^4$ cells/cm^2 using DMEM High Glucose supplemented with FBS (10 %), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and amphotericin B (2.5 µg/mL). Four hours after seeding, cells were transfected with the appropriate plasmid with the help of ExGen 500 according to the manufacturer's protocol.

pTriEx-1.1 without any inserts always served as negative control. After transfection, the cells were incubated for two days at 37 °C, 95 % humidity and 8.5 % CO_2.

CultureSlides or glass slides were washed two times with PBS and fixed in a solution of 1.8 % formaldehyde in PBS for 3 min. Afterwards, they were washed with a protecting solution (EUROIMMUN, Germany) and air-dried. CultureSlides were directly subjected to staining, glass slides were used for BIOCHIP production[122].

3.4.2 Evaluation of Immunofluorescence Substrates

For immunofluorescence staining, primary antibodies or sera were diluted in albumin/azide and incubated with the cells for 30 min at RT. In case of the incubation of BIOCHIPs, TITERPLANES™ (EUROIMMUN, Germany) were used. After the first incubation step, cells were rinsed with PBS/T and then washed in a PBS/T-containing chamber for 5 min at RT. Secondary antibodies were diluted in albumin/azide and incubated with the fixed cells for 30 min at RT, followed again by rinsing and washing with PBS/T. Then BIOCHIPs or CultureSlides were covered with a glass slide using covering medium. Examination of the stained cells was done with a Zeiss Axioskop 2 (Zeiss, Germany). Pictures were taken with a LuCam Camera (Lumenera Corporation, Canada) and the supplied Software LuCam Capture 5.0. For some experiments, the confocal microscope LSM 700 (Zeiss, Germany) and the appropriate software ZEN 2009 were used.

3.5 Westernblot Analysis

NuPAGE® 4-12 % Bis-Tris gels (Invitrogen, Germany) with a width of 1 mm were used for SDS-PAGE according to the manufacturer's instructions and with the supplied MES-buffer. Proteins were then blotted onto a nitrocellulose membrane (Purabind 02, GE-Healthcare Europe, Germany) in 60 min at 400 mA using NuPAGE® Transfer Buffer. Complete blotting was confirmed with a 10 min Ponceau S staining. After destaining with 50 mM Tris, membranes were blocked for 15 min at RT with blocking buffer (EUROIMMUN, Germany) under slight shaking. Antibodies and conjugates were diluted in Westernblot blocking buffer and incubated for 30 min at RT under slight shaking. Between the incubation steps of primary and secondary antibody, the membranes were washed three times with Westernblot washing buffer. After incubation with the secondary antibody, membranes were washed two times and then one time over 15 min under slight shaking. Westernblot substrate (EUROIMMUN, Germany) was utilised for staining of protein bands. The staining reaction was stopped with distilled water.

For Coomassie staining, NuPAGE® 4-12 % Bis-Tris gels (Invitrogen, Germany) with a width of 1 mm were used for SDS-PAGE. Then the gel was incubated with Coomassie destaining solution for 10 min at RT under slight shaking to achieve a fixation of the gel.

After discarding the destaining solution, staining was done with Coomassie brilliant blue G250 for 30 min at RT. For destaining, the Coomassie solution was exchanged against destaining solution. If necessary, the destaining solution was substituted with fresh one after some time. The gel was incubated in this solution until protein bands were clearly visible.

3.6 Production of Soluble Desmogleins in HEK 293T Cells

HEK 293T cells were seeded into Corning® HYPERFlasks (Omnilab-Laborzentrum, Germany) with a density of $7.5 \cdot 10^4$ cells/cm^2 in DMEM High Glucose supplemented with FBS (10 %), penicillin (100 U/mL), streptomycin (0.1 mg/mL) and amphotericin B (2.5 µg/mL). Four hours after seeding, cells were transfected with the appropriate plasmid with the help of ExGen 500. Then the cells were kept at 37 °C, 95 % humidity and 8.5 % CO_2 for 5-7 days.

For purification, cell culture supernatant was supplemented with 1 x TNIMCa and set to a pH of 8.0, followed by incubation at RT for 30 min. Then the supernatant was cleared by centrifuging. Purification was done with immobilised metal ion affinity chromatography (IMAC) on NiNTA Sepharose. For protein elution, increasing concentrations of imidazole were applied. After purification, a buffer exchange against Dsg-TBS/20 % Glycerol (in case of Dsg1) or Dsg-TBS (for Dsg3) was done. Purified proteins were stored at -20 °C or -80 °C, respectively.

3.7 Coupling of Antigens to Solid Phases

For coupling of proteins onto solid supports, the storage buffer of the proteins had to be exchanged against the respective coupling buffer by dialysis or gel filtration. Protein concentration was set to 0.5-1.0 µg/µL. Successful coupling was controlled via Westernblot or, in case of antibodies, via SDS-PAGE and subsequent Coomassie staining.

3.7.1 NHS-Activated Sepharose

For coupling of Dsg variants, NHS coupling buffer 1, blocking buffer 1 and washing buffer 1 were used. In case of antibodies, coupling buffer 2, blocking buffer 2 and washing buffer 2 were utilised.

The required amount of NHS-activated Sepharose was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter and washed with at least six gel volumes of ice cold 1 mM hydrochloric acid. Then the respective amount of protein was added, followed by a 30 min-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, the gel was washed with at least six volumes of blocking buffer, six volumes of washing buffer and four volumes of blocking buffer. Free NHS-groups were

blocked with two gel volumes of blocking buffer for 30 min at RT while rotating, followed by another washing with six volumes of washing buffer, six volumes of blocking buffer, six volumes of washing buffer and then six volumes of Dsg-TBS (in case of desmogleins) or PBS (four coupling of antibodies). For storage at 4 °C, 0.045 % NaN_3 was added to the slurry.

3.7.2 Aldehyde Agarose

The required amount of aldehyde agarose (AminoLink Plus Agarose) was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter and washed with at least six gel volumes of coupling buffer. Then the respective amount of protein and 50 mM of $NaCNBH_3$ were added, followed by a 6 h-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, the gel was washed with at least six volumes of coupling buffer, and four volumes of blocking buffer. Free aldehyde-groups were blocked with two gel volumes of blocking buffer and 50 mM $NaCNBH_3$ for 30 min at RT while rotating, followed by another washing with ten volumes of washing solution and six volumes of Dsg-TBS. For storage at 4 °C, Dsg-TBS was supplemented with 0.045 % NaN_3.

3.7.3 Tresyl-Activated Acrylic Resin

The required amount of tresyl acrylate (Toyopearl AF-Tresyl-650) was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter and washed with at least ten gel volumes of distilled water. Then the respective amount of protein was added, followed by a 2 h-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, the gel was washed with at least fifty volumes of Dsg-TBS. Free reactive groups on the resin were blocked with five gel volumes of blocking buffer for 1 h at RT while rotating, followed by another washing with eight volumes of Dsg-TBS. For storage at 4 °C, Dsg-TBS was supplemented with 0.045 % NaN_3.

3.7.4 Carboxy-Activated Acrylic Resin

The required amount of carboxy acrylate (Toyopearl AF-Carboxy-650) was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter and washed with at least ten gel volumes of distilled water and then with ten gel volumes of 0.5 M sodium chloride solution (pH 4.5-5). To activate the resin, four gel volumes of a fresh mixture of EDC (0.2 M) and NHS (50 mM) were incubated with the carboxy acrylate for 10 min at RT, before washing with four gel volumes of 0.5 M NaCl solution (pH 4.5-5). Then the respective amount of protein was added, followed by a 24 h-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, the gel was washed with at least ten

volumes of 5 mM calcium chloride solution, ten volumes of washing solution and ten volumes of Dsg-TBS. For storage at 4 °C, Dsg-TBS was supplemented with 0.045 % NaN_3.

3.7.5 Aldehyde Acrylic Resin

The required amount of aldehyde acrylate (Toyopearl AF-Formyl-650) was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter and washed with at least four gel volumes of distilled water and four volumes of coupling buffer. Then the respective amount of protein and 50 mM of $NaCNBH_3$ were added, followed by an over-night-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, free aldehyde groups were blocked with six gel volumes of blocking buffer and 50 mM $NaCNBH_3$ for 1 h at RT while rotating. Afterwards, the gel was washed with at least ten volumes each of 5 mM calcium chloride, washing solution, again 5 mM $CaCl_2$ and then Dsg-TBS. For storage at 4 °C, Dsg-TBS was supplemented with 0.045 % NaN_3.

3.7.6 Azlactone Acrylic Resin

The required amount of azlactone acrylate (UltraLink Biosupport) was transferred into a MoBiCol column (MoBiTec, Germany) with a 10 µm filter. The storage solution was removed and then the respective amount of protein was added, followed by a 1 h-incubation at RT. A rotator was used to prevent sedimentation of the slurry. After coupling, free reactive groups were blocked with ten gel volumes of NHS blocking buffer for 2.5 h at RT while rotating. Then the gel was washed with at least ten gel volumes of aldehyde washing buffer over 15 min, followed by 20 volumes of Dsg-TBS. For storage at 4 °C, Dsg-TBS was supplemented with 0.045 % NaN_3.

3.8 Adsorption of Antibodies Using Dsg Matrices

20 µL of Dsg matrices were washed three times with 100 µL Dsg-TBS each in a mini spin column (MoBiTec, Germany) before adding human sera (200 µL if not stated otherwise). The slurry was incubated for 30 min at RT on a rotator. Afterwards, the sera were removed by centrifugation.

For regeneration after the adsorption procedure, Dsg resins were washed with at least 3 x 200 µL of Dsg-TBS. Then 100 µL elution buffer were added and incubated for 2 min at RT. After removal of the elution buffer by centrifugation, the pH was raised with 100 µL neutralisation buffer, which was incubated with the matrices for 2 min at RT. The procedure of elution and neutralisation was repeated and then the adsorbers were washed again three times with 200 µL Dsg-TBS each.

3.9 Antibody Detection by ELISA

3.9.1 Detection of Specific IgG

Anti-Dsg1, anti-Dsg3 and anti-EBNA1 ELISA kits were obtained from EUROIMMUN and carried out according to the manufacturer's protocol. All samples were measured in duplicate. Evaluation of the results was done with a Tecan Sunrise^{TM} microplate reader and easyWIN fitting 6.0 (both Tecan, Germany). Measurement of the extinction was done at a wavelength of 450 nm and a reference wavelength of 620 nm. The amount of antibodies in the samples was calculated with the help of the provided controls.

Anti-Dsg1 and anti-Dsg3 ELISAs were also used for measuring the amount of anti-Dsg antibodies in rabbit sera. In these cases, an anti-rabbit IgG horseradish peroxidase (HRP) conjugate dissolved in 1 x Taubert buffer was used as secondary antibody instead of the supplied anti-human IgG HRP conjugate.

3.9.2 Analysis of IgG Concentration

MaxiSorp^{TM} plates (VWR International, Germany) were coated with an antibody directed against the human IgG Fc chain (1 µg/well) for 3 h at RT. After removal of the antibody solution, the wells were blocked with casein for 1 h at RT and washed with ELISA washing buffer (300 µL/well, 3 times, 30 s each). Samples and calibration specimen (EUROIMMUN, Germany) were diluted 1:1000 in casein sample buffer and incubated with the ELISA wells for 30 min at RT (100 µL/well). This was followed by another washing step as described above. As secondary antibody, a HRP-conjugate directed against human IgG F(ab')$_2$-fragments was used. The conjugate was diluted 1:20,000 in Taubert buffer and incubated for 30 min at RT (100 µL/well). After washing, each well was incubated with 100 µL ELISA substrate for 15 min at RT in the dark. The reaction was stopped with 100 µL/well stopping solution. The readout was carried out as described above.

3.10 Pathogenicity Assays

3.10.1 Anti-Dsg1 Antibodies

The cell fragmentation assay was done at the Institute of Dermatology and Venerology at the University of Lübeck. HaCaT cells, an immortalised human keratinocyte cell line[27], were grown in KGM2 supplemented with all supplied hormones and growth factors, $CaCl_2$ (1.2 mM), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). Cells were seeded into 12 well plates and grown to confluence at 37 °C, 95 % humidity and 8.5 % CO_2. Then the medium was exchanged against fresh one and pemphigus sera as well as negative controls (normal human sera) were diluted 1:5 till 1:25 into the medium. After incubation for addi-

tional 24 h, the cell culture supernatant was removed. Cells were washed three times with PBS before adding 500 µl dispase (1:2 dilution in PBS, final concentration 2.5 mg/mL) per well, followed by a 30 min incubation at 37 °C. After the cells had detached from the bottom of the culture plate, they were put under mechanical stress by pipetting the monolayer five times. Then 500 µl of 4 % formaldehyde were added. After incubation of this fixing solution for 5 min at RT, one drop of methyl violet 2B solution was used to stain the cell fragments (10 min at RT). In the meantime, petri dishes were filled with PBS. After staining of the cell fragments was complete, they were transferred into the petri dishes and photographed. The number of fragments was counted using ImageJ 1.45s.

To detect phosphorylation of p38 MAPK, HaCaT cells grown in DMEM High Glucose supplemented with FBS (10 %), penicillin (100 U/mL) and streptomycin (0.1 mg/mL) were seeded onto 24 well plates (Nunc, Germany) with a density of 10^5 cells per well. After incubation for 24 h at 37 °C, 95 % humidity and 8.5 % CO_2, the medium was exchanged and human sera were added in a 1:5 dilution, followed by another incubation step at 37 °C for 30 min. Then the cells were detached from the culture plate using a cell scraper and transferred into reaction tubes. After centrifuging the suspension for 5 min at 4 °C and 20,800 g, the supernatant was removed and the pellet resuspended in a small volume of PBS/U. Samples were analysed by Western blotting using an anti-p38 MAPK antibody and an anti-phospho-p38 MAPK antibody.

For observation of actin retraction, HaCaT cells were seeded onto BD Falcon™ CultureSlides (VWR International, Germany) with a density of $2.4 \cdot 10^4$ cells/cm^2 using DMEM High Glucose supplemented with FBS (10 %), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The cells were incubated for 24 h at 37 °C, 95 % humidity and 8.5 % CO_2 before exchanging the culture medium and adding human sera in a 1:20 dilution. After incubation for another 24 hours, CultureSlides were washed two times with PBS, one time with acetone and fixed in fresh acetone for 10 min. Then they were washed with protecting solution (EUROIMMUN, Germany), air-dried and subjected to immunofluorescence staining (see chapter 3.4.2) using phalloidin-TRITC.

3.10.2 Anti-Dsg3 Antibodies

HaCaT cells were seeded onto BD Falcon™ CultureSlides (VWR International, Germany) with a density of $2.4 \cdot 10^4$ cells/cm^2 using DMEM High Glucose supplemented with FBS (10 %), penicillin (100 U/mL) and streptomycin (0.1 mg/mL). The cells were incubated for 24 h at 37 °C, 95 % humidity and 8.5 % CO_2 before adding human sera to the medium in a 1:20 dilution. After another 48 h-cultivation, the cells were fixed and stained as described in chapter 3.4.2. For primary detection, a monoclonal antibody directed

against human Dsg3 was used (dilution: 1:50). As secondary antibody, an anti-mouse IgG cyanine 2 (Cy2) conjugate was applied (dilution: 1:200).

3.11 Software for Diagrams and Statistics

Microsoft® Office Excel 2003 was used for calculations and generations of diagrams. For statistical tests and calculations, SigmaPlot 12 was used.

4. Results

4.1 Properties of Different Adsorption Materials

4.1.1 Coupling Efficiency of Dsg1(ec)-His and Dsg3(ec)-His

There are many different solid supports available for immobilising proteins, peptides or small molecules. Most of them react with primary amino groups, carboxyl groups, aldehyde groups or sulfhydryl groups. To couple proteins via free carboxyl groups, the protein itself must be activated, for example by EDC and NHS[56]. This can lead to intra- or intermolecular reactions of the protein itself, which might be disadvantageous when handling big molecules such as Dsg1 and Dsg3. Aldehyde groups in proteins exist only in the carbohydrate chains of glycoproteins. Although Dsg1 and Dsg3 are glycosylated, the role of this modification for recognition by autoantibodies is not entirely clear. It has been reported that epitopes on pemphigus antigens are glycosylation independent[7,9], but the expression of deglycosylated desmogleins was done in presence of tunicamycin, which only abolishes N-glycosylation. However, on both Dsg1 and Dsg3 there are several potential O-glycosylation sites[25,134], which have not been investigated so far. Using aldehyde groups as targets for the coupling of desmogleins was, therefore, not considered. Finally, sulfhydryl groups are important for the formation of disulfide bonds, and, this way, contribute to the conformation of proteins. Since in case of pemphigus antigens the correct folding of the proteins is necessary for antibody binding, coupling via sulfhydryl groups seemed quite risky.

Out of the portfolio of coupling reactions, immobilisation via primary amines was considered most promising. Six different preactivated solid supports were chosen for preliminary coupling tests: NHS-activated Sepharose, Toyopearl Tresyl, Toyopearl Carboxy, Toyopearl Formyl, AminoLink Plus agarose and UltraLink Biosupport. NHS-Sepharose is crosslinked agarose carrying NHS-groups. Toyopearls and the UltraLink Biosupport consist of polyacrylamide and are activated by tresyl-groups (Toyopearl Tresyl), carboxy-groups (Toyopearl Carboxy), aldehyde groups (Toyopearl Formyl) or azlactone groups (UltraLink). AminoLink Plus agarose is crosslinked agarose carrying aldehyde groups.

Purified Dsg1(ec)-His and Dsg3(ec)-His were coupled to all these matrices with a density of 0.5 µg protein per µL solid support. This was the lowest possible coupling capacity stated by the manufacturers of the resins. Since Dsg1(ec)-His is barely stained by Coomassie blue, efficiency of the coupling reactions was monitored via Western blotting (Figure 6).

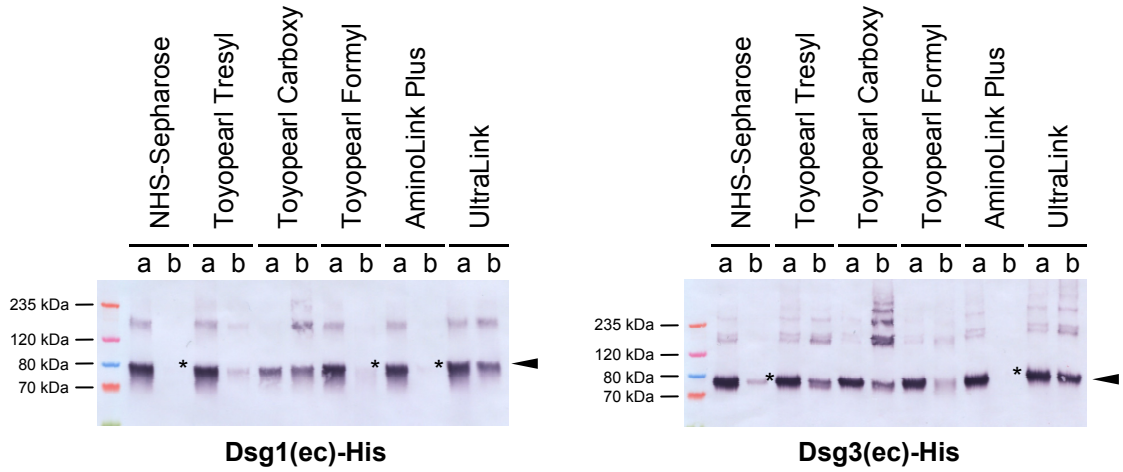

Figure 6: Recombinant desmogleins can be coupled to different solid supports
Dsg1(ec)-His and Dsg3(ec)-His were immobilised on different preactivated solid supports with a density of 0.5 µg protein per µL matrix. Control samples were taken before (a) and after coupling (b) for Westernblot analysis. Detection of the recombinant proteins was achieved with a monoclonal anti-His-tag antibody. The molecular weight standard is given on the left. Arrowheads show Dsg1(ec)-His and Dsg3(ec)-His, respectively. Most efficient coupling of the proteins is indicated by asterisks.

Samples were taken of the protein solutions before and after the reactions. They show that coupling onto NHS-Sepharose and AminoLink Plus agarose was very efficient in case of both antigens, since after coupling both desmogleins were hardly detectable anymore in the reaction mix. It can be assumed that it is possible to bind even more than 0.5 µg protein per µL to these solid supports. Dsg1(ec)-His was immobilised to a high extent onto Toyopearl Formyl, whereas after coupling of Dsg3(ec)-His to this matrix, there was still an considerable amount of antigen left. Reaction of both desmogleins with the UltraLink resin resulted in even more residual protein in the coupling solution, indicating a non-exhaustive reaction. Coupling to Toyopearl Carboxy led to the appearance of protein bands of high molecular weight in the blot analysis. This might be a result of insufficient washing of the matrix after activation with EDC and NHS. Remainders of these substances might have led to crosslinking of protein molecules with themselves.

4.1.2 Adsorption Performance of Different Dsg Matrices

Since coupling efficiency is not necessarily proportional to adsorption capacity of the immobilised protein, all Dsg matrices were subjected to adsorption of pemphigus sera. Different PF and PV sera were pooled and incubated with the Dsg matrices. A serum-matrix ratio of 2:1 was used. After adsorption, the amount of anti-Dsg1 and anti-Dsg3 antibodies was measured using anti-Dsg1 or anti-Dsg3 ELISA, respectively (Figure 7).

Concerning anti-Dsg1 IgG, their amount was reduced to 55-71 % after adsorption on Dsg1 Toyopearl Carboxy, Dsg1 Toyopearl Formyl, Dsg1 agarose and Dsg1 UltraLink. Adsorption on Dsg1 Toyopearl Tresyl resulted in a decrease of the anti-Dsg1 titre to 33 %

compared to the antibody level before adsorption. Dsg1 Sepharose was most efficient in binding anti-Dsg1 antibodies, leaving only 14 % of these IgG in the sera pool.

Least efficient Dsg3 matrices were Dsg3 Toyopearl Carboxy and Dsg3 Toyopearl Formyl with a reduction of the amount of anti-Dsg3 antibodies to 44 and 45 %, respectively. All other Dsg3 matrices were able to decrease the anti-Dsg3 antibody level to less than 10 %.

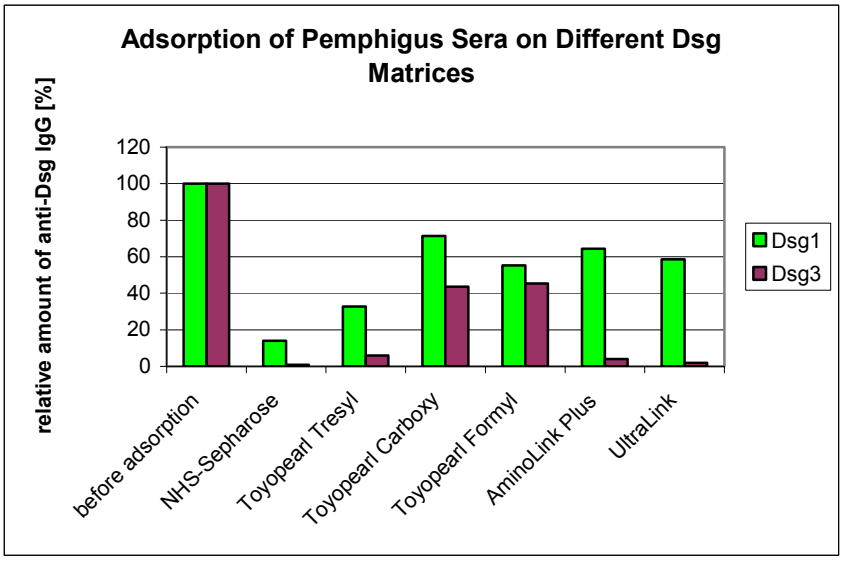

Figure 7: Pemphigus sera can be adsorbed on different Dsg matrices
A pool of 11 pemphigus sera (anti-Dsg1 titre: 379 U/ml, anti-Dsg3 titre: 336 RU/ml) was adsorbed on Dsg1(ec)-His and Dsg3(ec)-His coupled to different solid supports (n = 1). 50 µL sera pool were adsorbed on 25 µL matrix. After adsorption, the amount of anti-Dsg IgG in the flow-through was measured by anti-Dsg1 or anti-Dsg3 ELISA, respectively, and set in relation to the amount of anti-Dsg IgG prior adsorption (100 %).

In these preliminary experiments, NHS-Sepharose was by far the most effective solid support for immobilisation of Dsg1(ec)-His and binding of anti-Dsg1 IgG. In case of Dsg3(ec)-His, four matrices came into consideration: NHS-Sepharose, Toyopearl Tresyl, AminoLink Plus agarose and UltraLink Biosupport. To further specify the binding capacities of these four solid supports, coupling of Dsg3(ec)-His was repeated. Immobilisation of 0.5 µg protein per µL matrix did not reach the coupling capacity of the chosen materials, thus, the quantity of antigen subjected to the reaction was doubled for the following experiment. Then different amounts of a PV sera pool were processed with the Dsg3 adsorbers: five, ten and fifteen matrix volumes. Reduction of the anti-Dsg3 antibody level was confirmed via anti-Dsg3 ELISA (Figure 8).

Adsorption of five matrix volumes of PV sera pool led to nearly total removal of anti-Dsg3 IgG by Dsg3 Sepharose, Dsg3 Toyopearls and Dsg3 agarose. Dsg3 UltraLink was only capable of an antibody reduction to 31 %. After adsorption of ten volumes of sera pool, Dsg3 Sepharose and agarose decreased the amount of anti-Dsg3 IgG to less than 10 %, whereas adsorption capacity of the other resins was exceeded (> 25 % residual anti-Dsg3 IgG). Fifteen volumes of sera pool went beyond the binding capacity of all matrices.

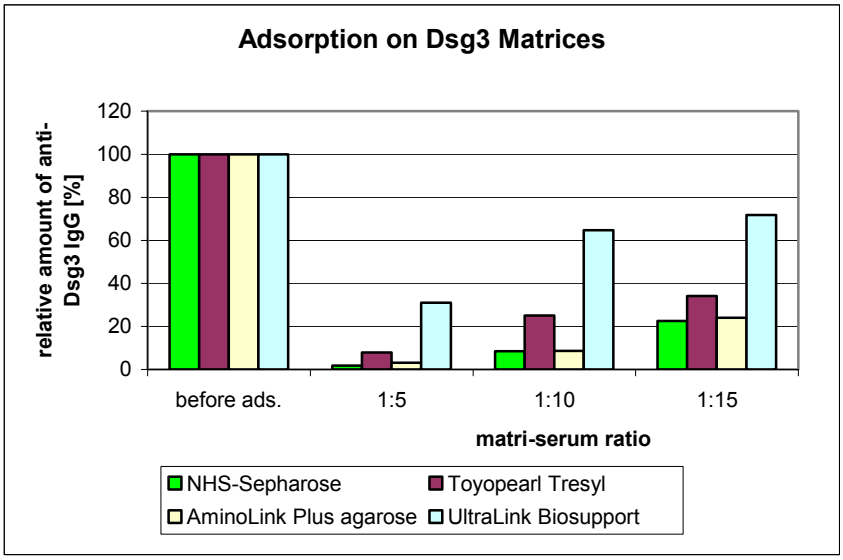

Figure 8: Large amounts of PV sera can be adsorbed on NHS-Sepharose and AminoLink Plus agarose
Dsg3(ec)-His was coupled onto different preactivated solid supports with a density of 1 µg protein per µL matrix. Different volumes of a pool of 15 PV sera (titre: 537 RU/ml) were adsorbed on these matrices (n = 1). After adsorption, the amount of Dsg3-antibodies in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of Dsg3-antibodies prior adsorption (100 %).

Dsg3 Sepharose and Dsg3 agarose were most efficient in binding anti-Dsg3 antibodies. Tresyl Toyopearls and UltraLink Biosupport were excluded from all further experiments.

Taking the results of the testing of matrices for both desmogleins together, NHS-activated Sepharose is a suitable activated solid support to couple Dsg1(ec)-His and Dsg3(ec)-His for immunoadsorption purposes. AminoLink Plus agarose was able to bind both antigens with high efficiency, but adsorption of autoantibodies was only successful in case of Dsg3(ec)-His. This was intriguing at first, because Dsg1(ec)-His and Dsg3(ec)-His are very similar to each other (sequence identity: 47.1 %, sequence similarity: 63.0 %) and coupling and adsorption conditions were identical. This oddity was cleared later on by the observation that Dsg1(ec)-His is more sensitive to changes in pH than Dsg3(ec)-His. Sodium cyanoborohydride, which is added to the coupling solution, is stored in a 1 M sodium hydroxide solution to prevent emission of hydrocyanic acid. Apparently, this raised the pH of the coupling solution in a way that it damaged the conformation of Dsg1(ec)-His. The problem could be solved by increasing the concentration of HEPES in the coupling buffer from 10 to 50 mM. This way, very good antibody binding of Dsg1 agarose could be achieved, too (see chapter 4.1.3).

4.1.3 Effect of Optimised Coupling Conditions

After NHS-Sepharose and AminoLink Plus agarose were chosen for further coupling experiments, the optimal protein load with respect to adsorption capacity had to be determined. Therefore, different amounts of the recombinant desmogleins were coupled to both solid supports (Figure 9).

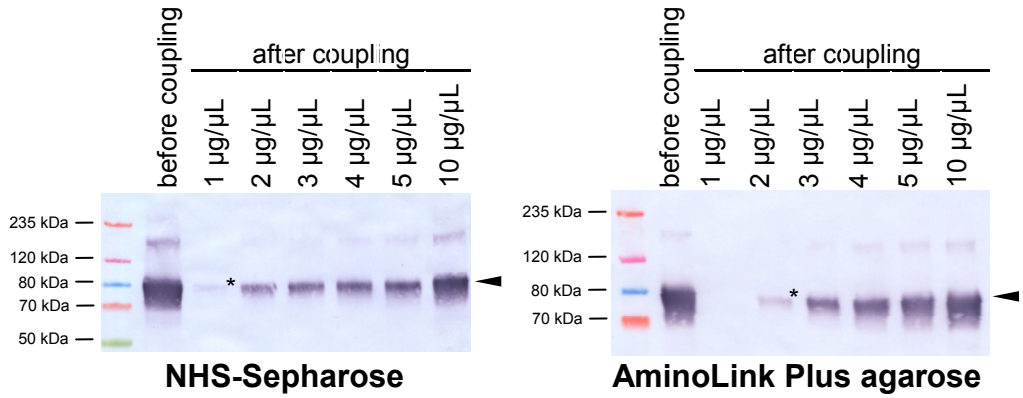

Figure 9: Dsg1(ec)-His can be coupled more efficiently to AminoLink Plus agarose than to NHS-activated Sepharose
Different amounts of Dsg1(ec)-His were immobilised on NHS-activated Sepharose and AminoLink Plus agarose. Control samples were taken before and after coupling for Westernblot analysis. Detection of the protein was achieved with a monoclonal anti-His-tag antibody. The molecular weight standard is given on the left. Arrowheads show Dsg1(ec)-His. Exceedance of coupling capacity is indicated by asterisks.

Concerning the coupling of Dsg1(ec)-His to NHS-Sepharose, it was observed that, even when coupling only 1 µg Dsg1(ec)-His per µL solid support, the reaction was not exhaustive. After coupling, there was still a small fraction of protein detectable by Western blotting. Consistent with this, after coupling of bigger amounts of Dsg1(ec)-His, the remaining protein portion was higher. The same effect occurred when immobilising the antigen on AminoLink Plus agarose, with the difference that saturation of the matrix was not reached until a coupling amount of 2 µg protein per µL solid support.

Although Dsg1(ec)-His could not be immobilised quantitatively, all of the differently loaded Dsg1 matrices were used for adsorption of PF sera pools.

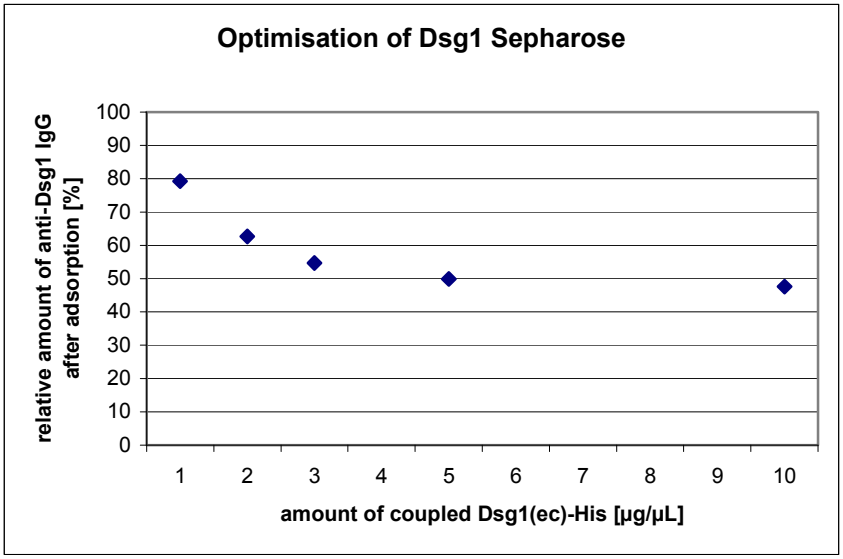

Figure 10: Optimisation of Dsg1 Sepharose
A pool of 7 PF sera (titre: 538 RU/ml) was adsorbed on differently loaded Dsg1 Sepharose (200 µL sera pool per 20 µL resin). After adsorption, the amount of anti-Dsg1 antibodies in the flow-through was measured by anti-Dsg1 ELISA and set in relation to the amount of anti-Dsg1 antibodies before adsorption (100 %). A protein load of 3 µg Dsg1(ec)-His per µL Sepharose is sufficient for adsorption of PF sera (n = 1).

Adsorption on Dsg1 Sepharose was inefficient compared with previous results (Figure 10). This is caused by the use of different sera pools. The pool utilised in this experiment had a very high anti-Dsg1 titre, which led to excess of the adsorption capacity of the resin.

The amount of anti-Dsg1 IgG differed only slightly after adsorption of the sera pool onto Sepharose coupled with 3 µg/µL (55 %), 5 µg/µL (50 %) and 10 µg/µL (48 %). Since there was no considerable improvement of the adsorption capacity even when much more protein was applied, a coupling concentration of 3 µg Dsg1(ec)-His per µL Sepharose was used from hereon.

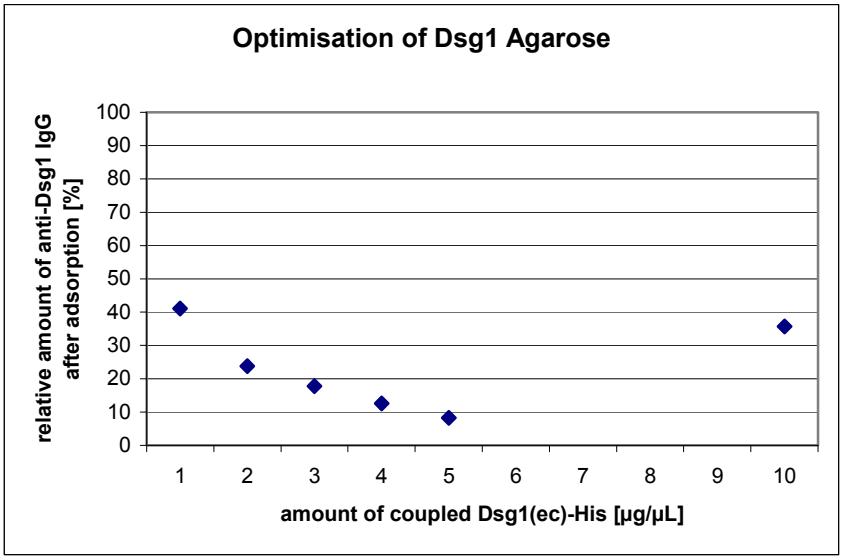

Figure 11: Optimisation of Dsg1 agarose
A pool of 9 PF sera (titre: 359 RU/ml) was adsorbed on differently loaded Dsg1 agarose (200 µL sera pool per 20 µL resin). After adsorption, the amount of anti-Dsg1 IgG in the flow-through was measured by anti-Dsg1 ELISA and set in relation to the amount of anti-Dsg1 IgG before adsorption (100 %). Best adsorption results were achieved with a protein load of 5 µg Dsg1(ec)-His per µL agarose (n = 1).

Adsorption on Dsg1 agarose was more efficient the more antigen had been used for coupling (Figure 11). The residual amount of anti-Dsg1 IgG decreased from 41 % after adsorption on agarose loaded with 1 µg/µL Dsg1 to 8 % after adsorption on agarose loaded with 5 µg/µL. When the coupling solution contained enough protein to load 10 µg per µL matrix, adsorption capacity dropped. 36 % of anti-Dsg1 antibodies were left in this sample. An explanation for this might be that steric hindrance occurred because of the high density of Dsg1(ec)-His molecules on the solid support. It is possible that the binding of anti-Dsg1 antibodies conceals epitopes of neighbouring molecules at this protein density.

The smallest amount of anti-Dsg1 IgG was detected after adsorption of the sera pool on Dsg1 agarose loaded with 5 µg protein per µL resin. For this reason and in light of the results of the optimisation of Dsg3 agarose, all further experiments were done with this coupling concentration.

Dsg3 matrices were optimised in the same way as Dsg1 resins: Dsg3(ec)-His was coupled to NHS-Sepharose and AminoLink Plus agarose using different protein-matrix ratios

and then these solid phases were subjected to adsorption. This time, an artificial sera pool (see chapter 3.2) was used to minimise deviations between different experiments.

The results of the analysis of the coupling procedures (Figure 12) show that loading capacity of NHS-Sepharose was exceeded when 1 µg Dsg3(ec)-His was added to 1 µL resin. The residual amount of protein after coupling increased with the applied starting amount. The same effect was observed concerning the immobilisation on AminoLink Plus agarose, but the antigen was detectable in the reaction mix only when 3 µg/µL or more where immobilised. Therefore, capacity of this solid support for coupling of Dsg3(ec)-His is higher than that of NHS-Sepharose.

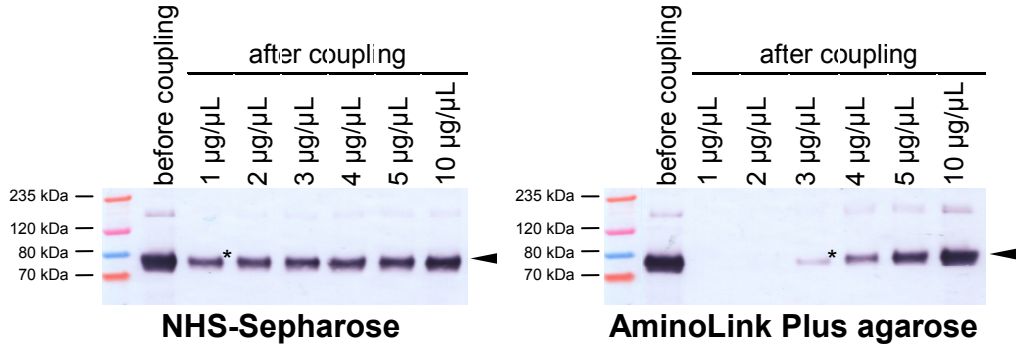

Figure 12: Dsg3(ec)-His can be coupled more efficiently to AminoLink Plus agarose than to NHS-activated Sepharose
Different amounts of Dsg3(ec)-His were immobilised on NHS-activated Sepharose and AminoLink Plus agarose. Control samples were taken before and after coupling for Westernblot analysis. Detection of the protein was achieved with a monoclonal anti-His-tag antibody. The molecular weight standard is given on the left. Arrowheads shows Dsg3(ec)-His. Exceedance of coupling capacity is indicated by asterisks.

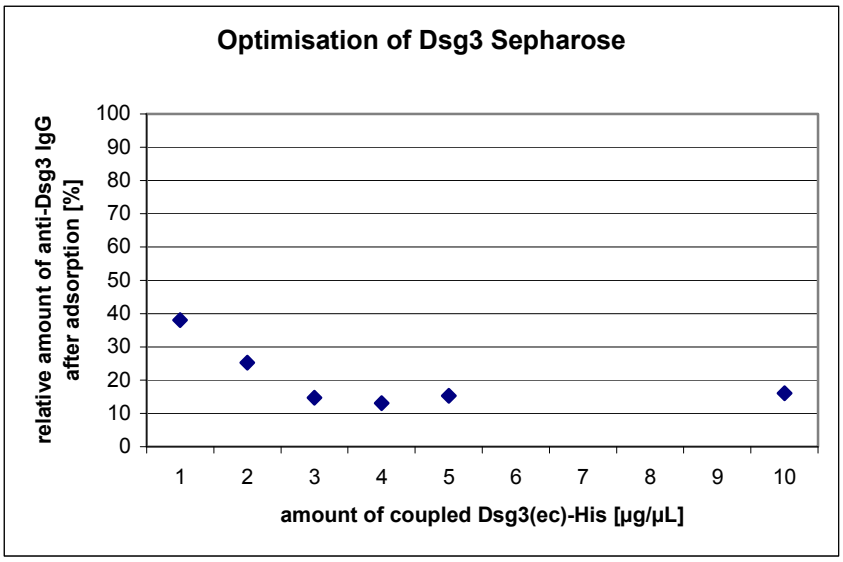

Figure 13: Optimisation of Dsg3 Sepharose
The artificial PV pool was adsorbed on differently loaded Dsg3 Sepharose (200 µL sera pool per 20 µL resin). After adsorption, the amount of anti-Dsg3 IgG in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of anti-Dsg3 IgG before adsorption (100 %). A protein load of 3 µg Dsg1(ec)-His per µL Sepharose is sufficient for adsorption of PF sera (n = 1).

Increasing amounts of protein led to increasing adsorption capacity of Dsg3 Sepharose (Figure 13). The residual anti-Dsg3 titre dropped from 38 % to 15 % when protein concen-

tration was raised from 1 µg/µL to 3 µg/µL. Sepharose loaded with 4, 5 or 10 µg Dsg3 per µL matrix adsorbed anti-Dsg3 IgG to a remain of 13 %, 15 % and 16 %, respectively. Analogous to Dsg1 Sepharose, the optimal ratio between protein consumption and adsorption efficiency was reached at a starting concentration of 3 µg Dsg3(ec)-His per µL Sepharose.

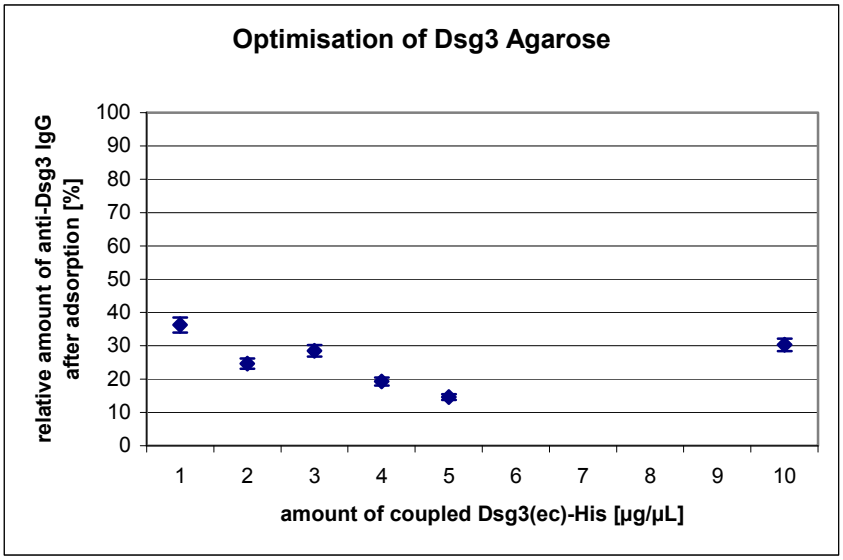

Figure 14: Optimisation of Dsg3 Agarose
The artificial PV pool was adsorbed on differently loaded Dsg3 agarose (200 µL sera pool per 20 µL resin). After adsorption, the amount of anti-Dsg3 IgG in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of anti-Dsg3 IgG before adsorption (100 %). Mean values of two independently conducted experiments are diagrammed. Indicated by error bars are standard deviations. Best adsorption results were achieved with a protein load of 5 µg Dsg1(ec)-His per µL agarose.

Figure 14 shows the results of the adsorption of the artificial sera pool on Dsg3 agarose. The residual portion of anti-Dsg3 antibodies dropped with increasing protein concentrations (1 to 5 µg/µL) from 36 % to 15 %, with one exception: At a starting concentration of 3 µg Dsg3(ec)-His per µL agarose, some kind of "pessimum" of the coupling reaction seemed to occur. The amount of anti-Dsg3 IgG that were left after adsorption was with 29 % higher than after adsorption on Dsg3 agarose produced with 2 µg or 4 µg protein per µL solid support (27 % and 19 %, respectively). Repetition of the experiment yielded the same results. Comparable to the adsorption on Dsg1 agarose, a protein load of 10 µg per µL resin decreased the antibody binding capacity. In this case, 30 % of anti-Dsg3 IgG remained. A coupling concentration of 5 µg Dsg3 per µL agarose was most efficient in adsorbing anti-Dsg3 IgG, therefore, this amount was applied for further experiments.

After determining the optimal Dsg loading for NHS-activated Sepharose and AminoLink Plus agarose, adsorption was repeated with the artificial sera pools to show reproducibility and to point out differences between the two solid supports. The PF pool was adsorbed on Dsg1 Sepharose and Dsg1 agarose, whereas the PV pool was processed with Dsg3 Sepharose and Dsg3 agarose (Figure 15).

The remaining anti-Dsg titre ranged from 14 % to 26 % compared to the amount of IgG before adsorption. Therefore, it could be shown that anti-Dsg antibodies can be removed by using Dsg1(ec)-His or Dsg3(ec)-His coupled to both resins. AminoLink Plus agarose was slightly more efficient than NHS-Sepharose. Although this was highly significant ($p < 0.005$ and $p < 0.001$, respectively), both activated solid supports were used for further experiments, because NHS-Sepharose has some advantages with respect to the coupling procedure (less protein, faster, no toxic reagents).

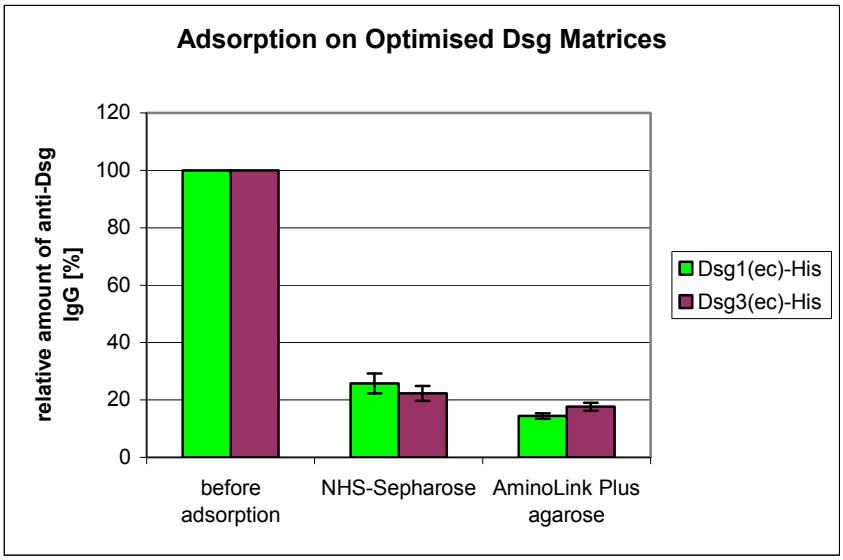

Figure 15: Adsorption on optimised Dsg1 and Dsg3 matrices is highly efficient
Dsg1(ec)-His and Dsg3(ec)-His were coupled under optimised conditions to NHS-Sepharose and AminoLink Plus agarose. Afterwards, the artificial PF sera pool was adsorbed on Dsg1 matrices and the artificial PV sera pool was processed with Dsg3 matrices (200 µL sera pool per 20 µL adsorber). After adsorption, the amount of anti-Dsg1 and anti-Dsg3 IgG in the flow-through was measured by anti-Dsg1 or anti-Dsg3 ELISA, respectively, and set in relation to the amount of IgG before adsorption (100 %). Mean values of three independent experiments are diagrammed. Indicated by error bars are standard deviations.

4.1.4 Specificity of the Prototypic Immunoadsorbers

After proving the principle of removing anti-Dsg antibodies via adsorption on different Dsg matrices, it was very important to know if this adsorption was specific. Specificity means that

a) adsorption of anti-Dsg antibodies is caused by the recombinant desmogleins and not by the solid supports, and that
b) Dsg matrices do only bind anti-Dsg antibodies, but not immunoglobulins directed against other target proteins.

To address this issue, both NHS-Sepharose and AminoLink Plus agarose were loaded with Dsg1(ec)-His on the one hand and blocked with ethanolamine or Tris on the other hand (empty Sepharose/agarose). Blocking the solid supports was done to saturate amino reactive groups on the surface of the carbohydrate beads. This way, the resins were made inert without coupling any protein. After generating these matrices, the artificial PF sera

pool was adsorbed on them and the amount of IgG was determined by ELISA as usual (Figure 16).

The level of anti-Dsg1 antibodies was lowered to 25 % and 15 % after adsorption on Dsg1 Sepharose and agarose, respectively, but only to 94 % after adsorption on blocked resins. In both cases, this difference between Dsg1 matrix and blocked matrix was highly significant ($p < 0.001$). Therefore, anti-Dsg1 antibodies in this sera pool specifically bind Dsg1(ec)-His.

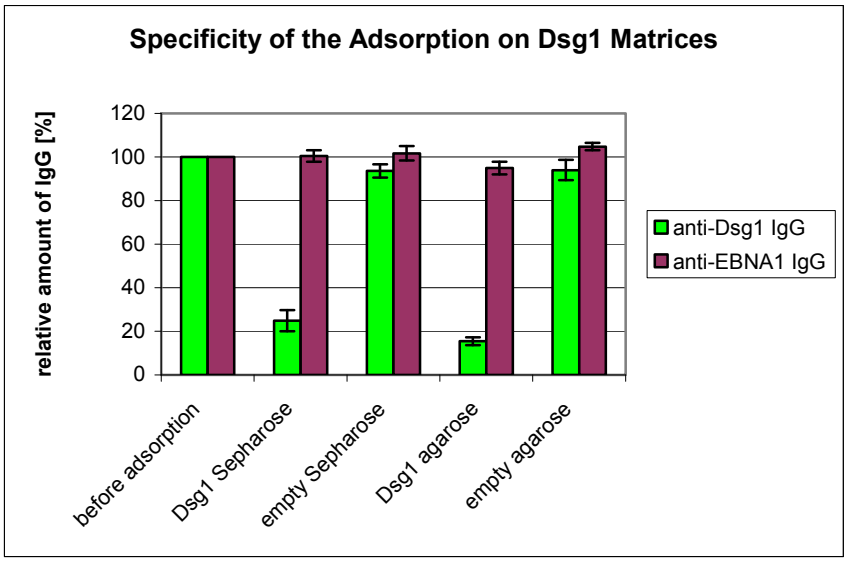

Figure 16: Adsorption on Dsg1 matrices is specific
Dsg1(ec)-His was coupled to NHS-Sepharose with a density of 3 µg protein per µL solid support and to AminoLink Plus agarose with a density of 5 µg protein per µL solid support. As controls, empty Sepharose and agarose were used. Then the artificial PF sera pool was adsorbed on these matrices. Samples were taken before and after adsorption and were analysed by anti-Dsg1 ELISA and anti-EBNA1 ELISA. The amount of IgG after adsorption was set in relation to the amount of IgG before adsorption (100 %). Mean values of three independently conducted experiments are displayed. Indicated by error bars are standard deviations.

The level of anti-EBNA1 antibodies was measured in parallel. EBNA1 is the main antigen of Epstein-Barr virus (EBV), a γ-herpes virus. Antibodies against EBNA1 are suited as specificity control, because 95 % of all adults are seropositive with respect to EBV and most of them possess anti-EBNA1 antibodies[81]. The experiment displayed in Figure 16 shows that the titre of anti-EBNA1 IgG did not change significantly despite adsorption ($p > 0.05$). This led to the conclusion that Dsg1 matrices as well as empty matrices do not bind pemphigus unrelated IgG.

These results were confirmed by observing the overall levels of IgG in the course of the adsorption procedure. For this experiment, a pool of ten PF sera was processed with Dsg1 matrices. The artificial sera pool was not used because of its elevated IgG level compared to normal human serum. The amount of whole IgG as well as anti-Dsg1 IgG was determined by ELISA before and after adsorption (Figure 17). Consistent with previous experiments, the portion of anti-Dsg1 antibodies dropped due to adsorption on Dsg1 matrices

to 12 % and 2 %. The total level of IgG did not change (97 % and 99 %). This showed again that Dsg1 matrices do not bind IgG apart from anti-Dsg1 antibodies.

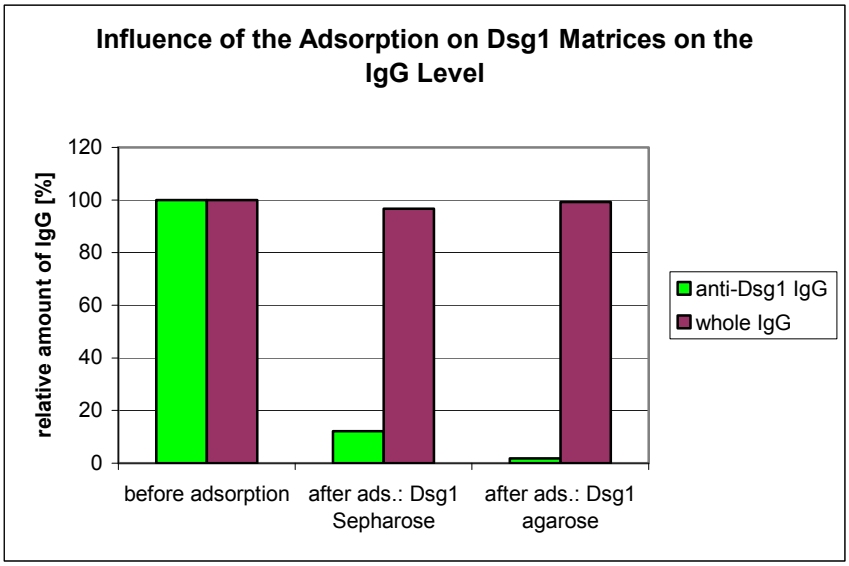

Figure 17: Adsorption on Dsg1 matrices does not influence the concentration of total IgG
A pool of 10 PF sera (titre: 324 RU/ml) was adsorbed on Dsg1 Sepharose and Dsg1 agarose (n = 1). Samples were taken before and after adsorption and were analysed by anti-Dsg1 ELISA and anti-human-IgG ELISA. The amount of IgG after adsorption was set in relation to the amount of IgG before adsorption (100 %).

Concerning specificity of Dsg3 adsorbers, experiments were conducted in the same manner. First, Dsg 3 matrices and empty matrices were used for adsorption of the artificial PV sera pool. The levels of anti-Dsg3 and anti-EBNA1 IgG were determined before and after adsorption (Figure 18). Processing on Dsg3 matrices reduced the amount of anti-Dsg3 antibodies to 21 % and 15 %, whereas adsorption on blocked solid supports led to a decrease of this antibody level to 90 % in both cases. Again, the difference in adsorption capacity of Dsg3 matrices and blocked matrices is highly significant ($p < 0.001$).

Adsorption of anti-EBNA1 IgG to Dsg3 matrices did not occur ($p > 0.05$). Therefore, Dsg3 matrices are specific. This was confirmed by observing the amount of overall IgG before and after adsorption on these matrices (Figure 19). Anti-Dsg3 antibodies were removed from a pool of 15 PV sera via adsorption on Dsg3 Sepharose and Dsg3 agarose. The remaining amount of anti-Dsg3 IgG was 4 % and 2 %. In contrast to this, the total of IgG did not change due to adsorption on Dsg3 matrices (96 % and 98 %). It can be concluded that Dsg3 adsorbers do not bind IgG apart from Dsg3 specific ones.

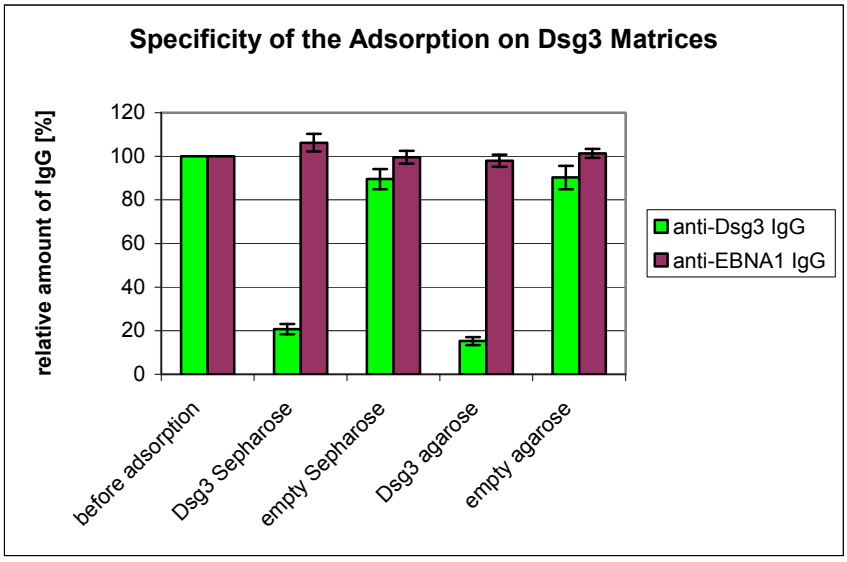

Figure 18: Adsorption on Dsg3 matrices is specific
Dsg3(ec)-His was coupled to NHS-Sepharose with a density of 3 µg protein per µL solid support and to AminoLink Plus agarose with a density of 5 µg protein per µL solid support. As controls, empty Sepharose and agarose were used. Then the artificial PV sera pool was adsorbed on these matrices. Samples were taken before and after adsorption and were analysed by anti-Dsg3 ELISA and anti-EBNA1 ELISA. The amount of IgG after adsorption was set in relation to the amount of IgG before adsorption (100 %). Mean values of three independently conducted experiments are displayed. Indicated by error bars are standard deviations.

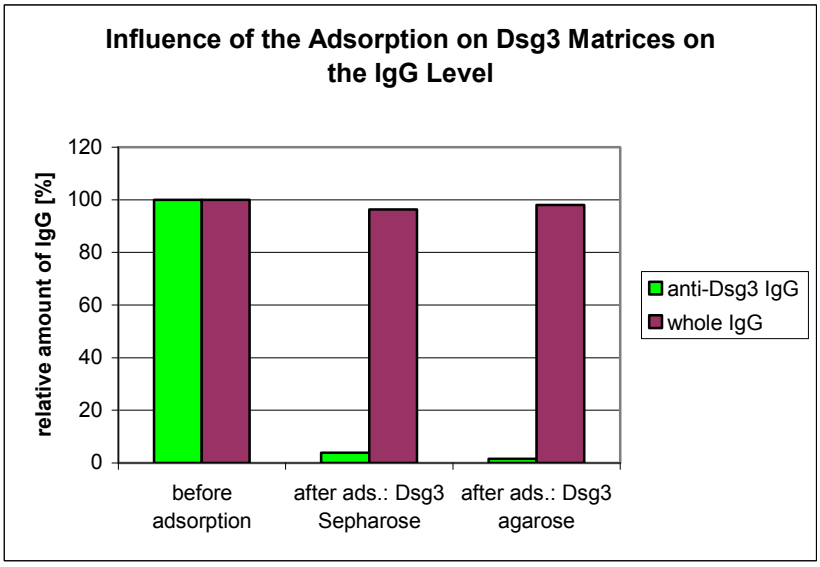

Figure 19: Adsorption on Dsg3 matrices does not influence the concentration of total IgG
A Pool of 15 PV sera (titre: 309 RU/ml) was adsorbed on Dsg3 Sepharose and Dsg3 agarose (n = 1). Samples were taken before and after adsorption and were analysed by anti-Dsg3 ELISA and anti-human-IgG ELISA. The amount of IgG after adsorption was set in relation to the amount of IgG before adsorption (100 %).

4.1.5 Stability of Dsg Matrices during Regeneration

Eukaryotic expression of proteins and their purification is expensive. It would be advantageous to produce adsorbers that can be regenerated, both for use in research and in therapy. The most common procedure to remove antibodies from their targets is elution with an acidic glycine buffer. This method was applied to Dsg1 and Dsg3 matrices. Artificial sera pools were processed with the adsorbers. Then Dsg matrices were regenerated and

again used for adsorption. This way, five adsorptions with fresh sera pool were done altogether.

Dsg1 Sepharose and Dsg1 agarose behave very similar with respect to their adsorption capacity (Figure 20). The residual amounts of anti-Dsg1 antibodies left in the artificial sera pool after adsorption increased by 30 % (from 25 to 55 %) in case of Dsg1 Sepharose and by 39 % (from 21 to 60 %) in case of Dsg1 agarose. Thereby, reduction of adsorption capacity was not continuous. There was a big leap between the first and second adsorption instead, whereas the differences between adsorption steps two till five were smaller.

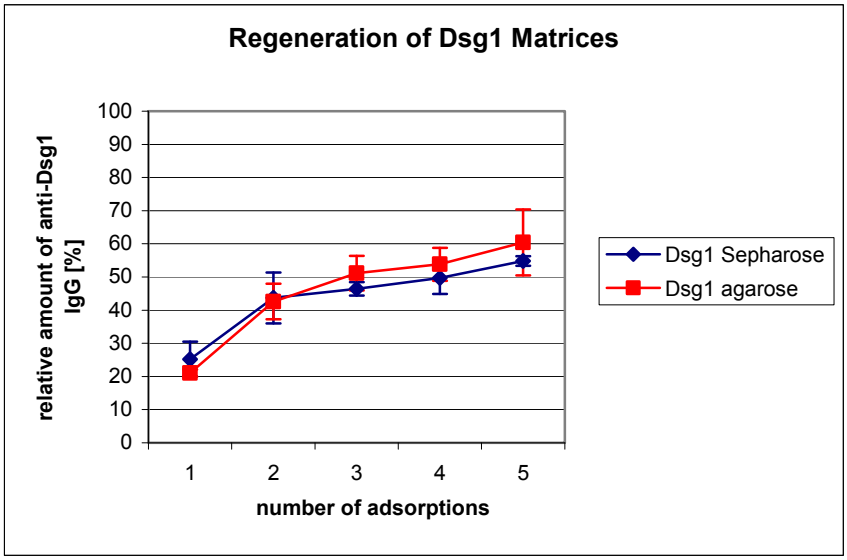

Figure 20: Loss of adsorption capacity upon regeneration of Dsg1 matrices
Dsg1 Sepharose and Dsg1 agarose were used for five adsorptions of the artificial PF pool. Between the adsorption steps, Dsg1 matrices were regenerated with a glycine buffer (pH 2.5). After each adsorption, the amount of anti-Dsg1 IgG in the flow-through was measured by anti-Dsg1 ELISA and set in relation to the amount of anti-Dsg1 IgG before adsorption (100 %). Mean values of two independently conducted experiments are shown. Indicated by error bars are standard deviations.

Regarding Dsg3 adsorbers (Figure 21), the variation between the residual amount of anti-Dsg3 antibodies after the first and fifth adsorption was with 11 % (19 to 30 %) and 12 % (16 to 28 %), respectively, much smaller compared to Dsg1 adsorbers. Again, differences between Sepharose and agarose were negligible.

Since regeneration of Dsg3 matrices worked well over five cycles, reuse over 20 cycles was tried next (Figure 22). This is about the number of cycles necessary to treat one patient with conventional IgG adsorbers (one session). The results of the first five adsorptions were comparable to previous experiments (Figure 21). Unfortunately, capacity of the Dsg3 matrices decreased with ongoing regenerations, meaning that the residual portion of anti-Dsg3 antibody increased from step to step. After 14 adsorption and regeneration cycles, the binding capacity of the matrices reached a plateau, where around 45 % of the anti-Dsg3 antibodies remained in the sera pool.

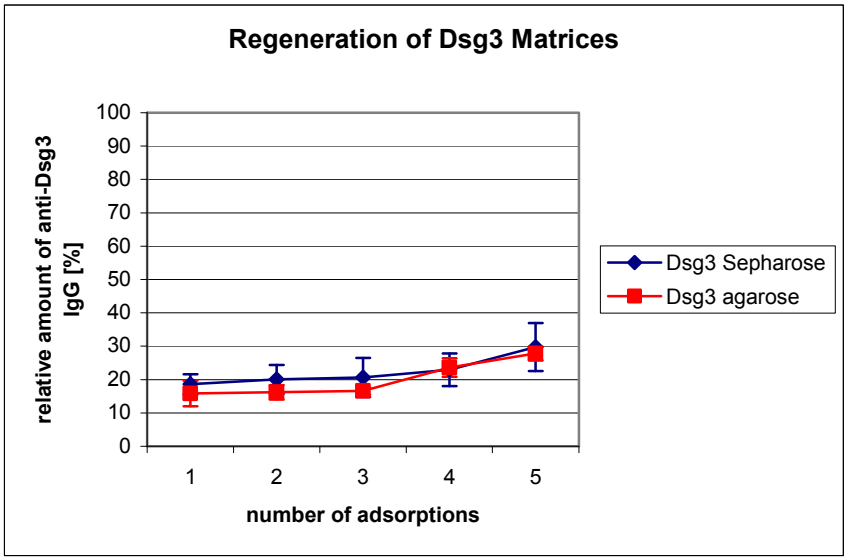

Figure 21: Dsg3 matrices can be regenerated
Dsg3 Sepharose and Dsg3 Agarose were used for five adsorptions of the artificial PV pool. Between the adsorption steps, Dsg3 matrices were regenerated with glycine buffer (pH 2.5). After each adsorption, the amount of anti-Dsg3 IgG in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of anti-Dsg3 IgG before adsorption (100 %). Mean values of two independently conducted experiments are shown. Indicated by error bars are standard deviations.

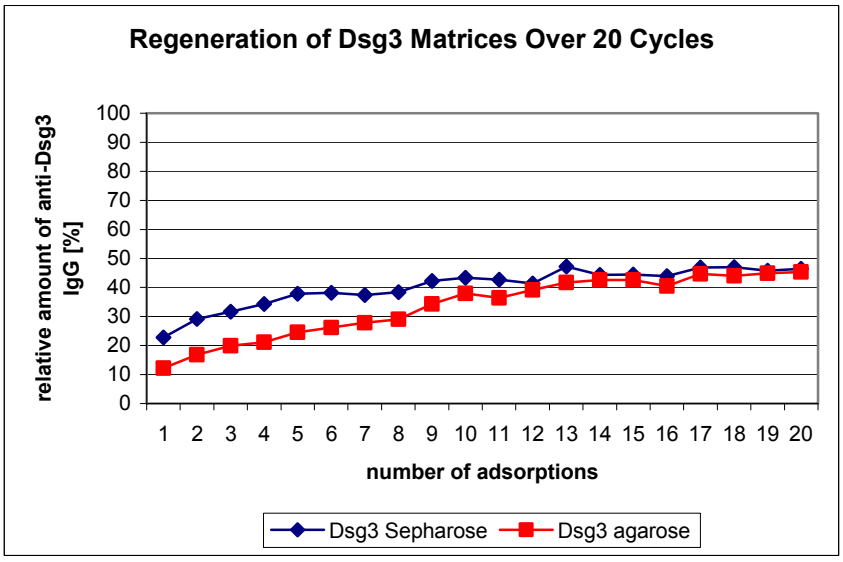

Figure 22: Antibody binding capacity decreases upon regeneration of Dsg3 matrices over 20 cycles
Dsg3 Sepharose and Dsg3 agarose were used for 20 adsorptions of the artificial PV pool (n = 1). Between the adsorption steps, Dsg3 matrices were regenerated with glycine buffer (pH 2.5). After each adsorption, the amount of anti-Dsg3 IgG in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of anti-Dsg3 IgG before adsorption (100 %).

Taken all findings together, acidic regeneration of Dsg matrices is possible, but not to a satisfying level. Other elution methods like high salt buffers or triethanolamine did not improve these results. Stabilising the antigens via intramolecular crosslinking was also tried, but was not successful.

There are three possible reasons for the drop of performance of desmoglein adsorbers:

I. The antigens or parts of them are released from the solid supports.

II. Anti-Dsg antibodies are eluted insufficiently and block the binding of other antibodies.

III. Certain conformational epitopes are destroyed in the course of regeneration.

To rule out the first possibility, a monoclonal anti-His antibody was adsorbed five times on all matrices. However, the amount of deployed antibody was to low to get definite results. Due to the high cost of this method, I refrained from repeating the experiment. Instead, adsorptions of anti-Dsg rabbit sera were done. They recognise linear epitopes, therefore, repeated processing of these sera with regenerated Dsg matrices should yield equal results as long as the antigens do not leave the solid support.

Adsorption of an anti-Dsg1 rabbit serum on Dsg1 matrices is displayed in Figure 23. Over five cycles, extinction of the flow through in ELISA analysis increased slightly from 5 % to 9 % in case of Sepharose and from 6 % to 19 % in case of agarose. From these results, it cannot be concluded that Dsg1 is not cleaved from the resins, but it is obvious that the big deficit in matrix longevity, that was observed when processing the artificial PF pool, cannot be caused by antigen loss alone.

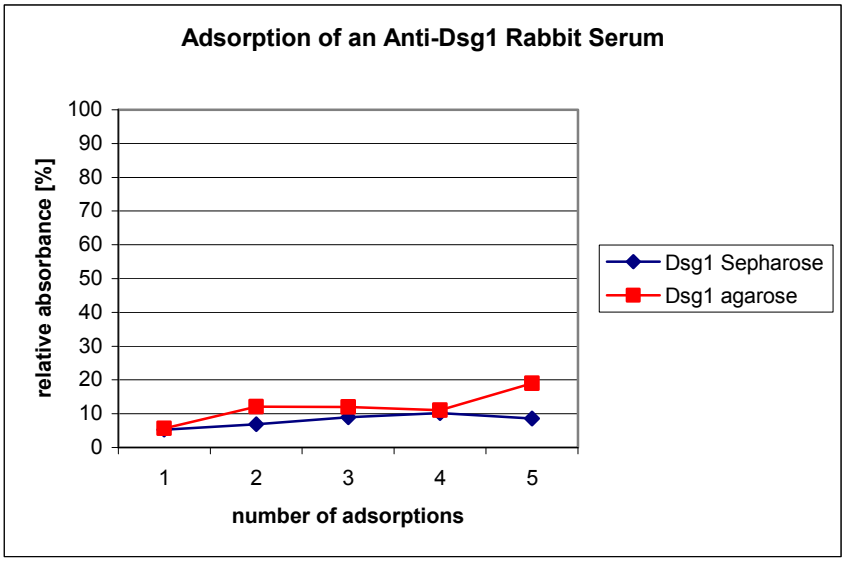

Figure 23: Stability of Dsg1 on the solid supports
Dsg1 Sepharose and Dsg1 agarose were used for 5 adsorptions of an anti-Dsg1 rabbit serum (n = 1). Between adsorption steps, Dsg1 matrices were regenerated with glycine buffer (pH 2.5). After each adsorption, the amount of rabbit anti-Dsg1 IgG in the flow-through was measured by anti-Dsg1 ELISA. The absorbances of the samples taken after individual adsorption steps were set in relation to the absorbance before adsorption (100 %).

To test if anti-Dsg1 antibodies were insufficiently eluted, the following experimental setup was conducted: Two aliquots of each Dsg1 matrix were compared with respect to their adsorption capacity. One aliquot was used directly for adsorption of the artificial PF sera pool. On the other one, the serum of a healthy blood donor was adsorbed four times with intermediate and subsequent regeneration, before the artificial PF sera pool was processed. This way, influences of serum components and the regeneration procedure on ad-

sorber matrices could be analysed while ruling out incomplete elution of anti-Dsg1 IgG, because the serum of the healthy blood donor did not contain such antibodies.

The results of this experiment are shown in Figure 24. The residual amount of anti-Dsg1 antibodies after adsorption on fresh Dsg1 matrices was with 35 % and 15 % considerably lower than after adsorption on the regenerated matrices, that left 56 % and 45 % of the anti-Dsg1 IgG in the sera pool. In fact, these results were well comparable to those obtained by reusing Dsg1 matrices five times for adsorption of the artificial PF pool. This shows that the lack of reusability is not due to insufficient elution of anti-Dsg1 antibodies. Therefore, the hypothesis that some epitopes are destroyed by the regeneration procedure is the most likely one for the explanation of the loss of binding capacity upon regeneration of Dsg1 adsorbers.

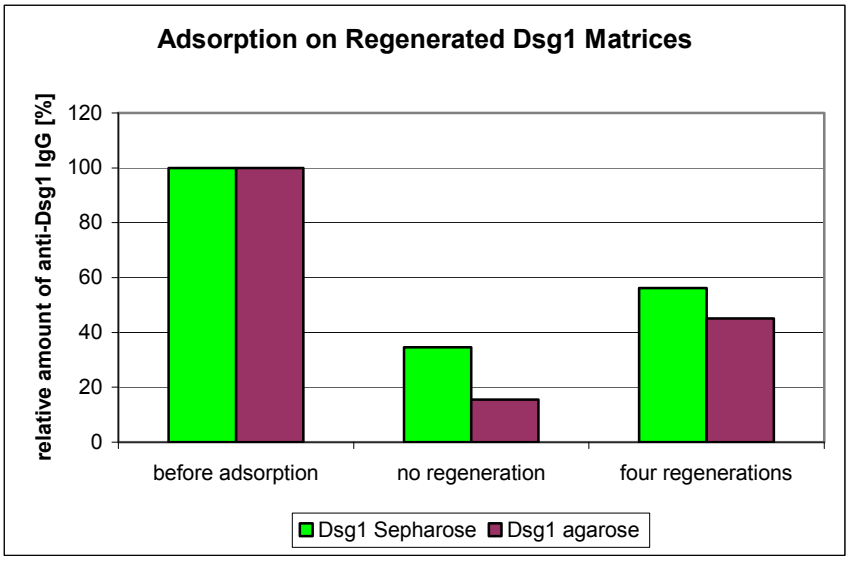

Figure 24: Loss of binding capacity of Dsg1 matrices is not caused by insufficient elution
One aliquot of each Dsg1 matrix was subjected to direct adsorption of the artificial PF sera pool (no regeneration), another aliquot was used for four adsorptions of a serum of a healthy blood donor and one adsorption of the artificial PF pool (four regenerations) (n = 1). Between the adsorption steps, antibodies were eluted with glycine buffer (pH 2.5). After each adsorption of the PF pool, the amount of anti-Dsg1 IgG in the flow-through was measured by anti-Dsg1 ELISA and set in relation to the amount of IgG before the adsorption (100 %).

Concerning Dsg3 adsorbers, experiments were conducted in the same way. Figure 25 shows the results of the adsorption of an anti-Dsg3 rabbit serum. Relative absorbance in the anti-Dsg3 ELISA of the flow through increased from 42 % to 45 % in both cases. Compared to the adsorption of the artificial PV sera pool (Figure 21), loss of adsorption capacity is smaller. Similar to the adsorption of a rabbit serum on Dsg1 matrices, it cannot be absolutely excluded by these results that Dsg3(ec)-His dissociated from the solid support. But if a fraction of the antigen leaves the resin, the percentage is very low.

Next, insufficient elution of anti-Dsg3 antibodies had to be investigated by adsorbing the serum of a healthy blood donor on Dsg3 matrices. After four cycles of adsorption and regeneration, the artificial PV pool was processed with these matrices. The flow through

was compared with a flow through of the PV sera pool adsorbed on fresh Dsg3 matrices (Figure 26).

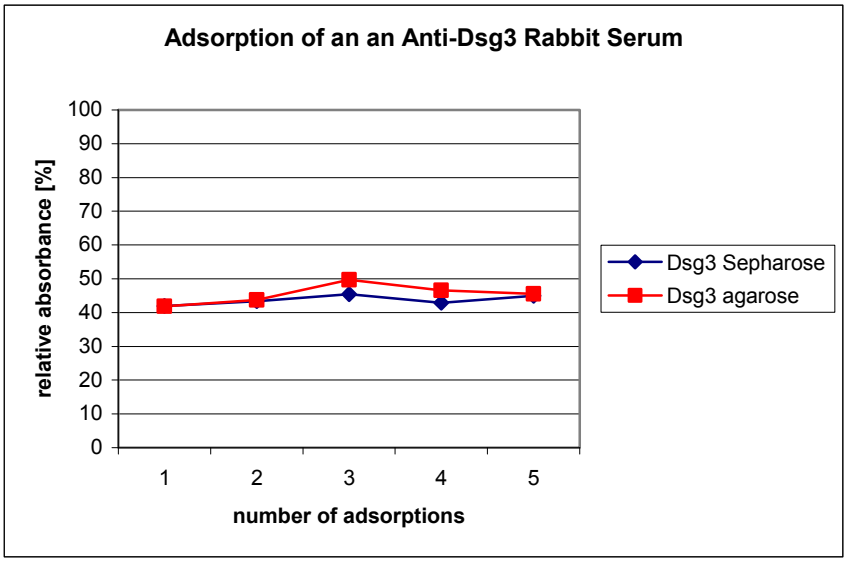

Figure 25: Stability of Dsg3 on the solid supports
Dsg3 Sepharose and Dsg3 agarose were used for 5 adsorptions of an anti-Dsg3 rabbit serum (n = 1). Between the adsorption steps, Dsg1 matrices were regenerated with glycine buffer (pH 2.5). After each adsorption, the amount of rabbit anti-Dsg3 IgG in the flow-through was measured by anti-Dsg1 ELISA. The absorbances of the samples taken after individual adsorption steps were set in relation to the absorbance before adsorption (100 %).

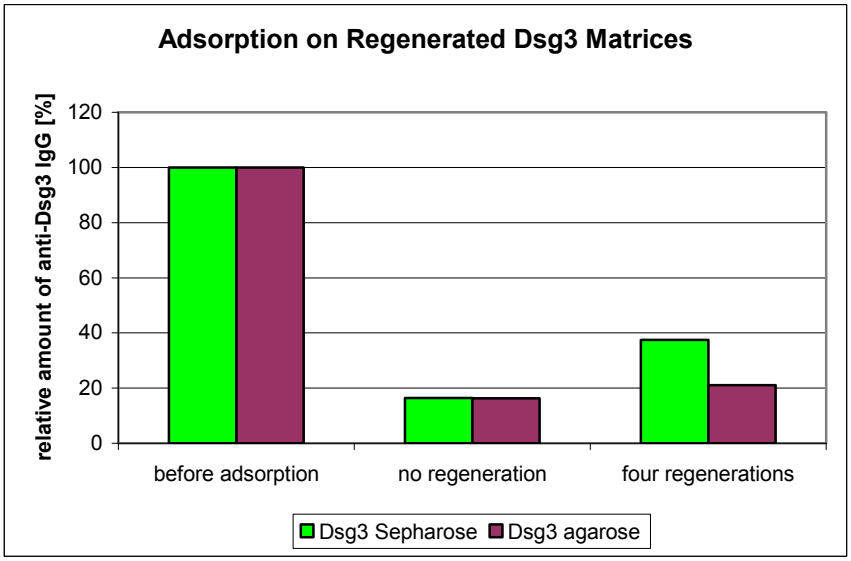

Figure 26: Loss of binding capacity of Dsg3 matrices is not caused by insufficient elution
One aliquot of each Dsg3 matrix was subjected to direct adsorption of the artificial PV sera pool (no regeneration), another aliquot was used for four adsorptions of a serum of a healthy blood donor and one adsorption of the artificial PV pool (four regenerations) (n = 1). Between the adsorption steps, antibodies were eluted with glycine buffer (pH 2.5). After each adsorption of the PV pool, the amount of anti-Dsg3 IgG in the flow-through was measured by anti-Dsg3 ELISA and set in relation to the amount of IgG before adsorption (100 %).

Dsg3 Sepharose showed a 21 % difference in adsorption capacity of fresh matrix compared to regenerated matrix (16 % to 37 %). This is in line with the results obtained from repeated adsorption of the artificial PV sera pool (Figures 21 and 22). The performance of regenerated Dsg3 agarose was nearly as good as the one of fresh matrix (16 % and 21 %).

Both observations exclude insufficiently eluted anti-Dsg3 antibodies as cause for the loss of adsorption capacity of Dsg3 matrices. Therefore, during acidic regeneration of Dsg3 matrices, a subset of epitopes seems to be destroyed.

4.2 Adsorption on Dsg Fragments

Dsg1(ec)-His and Dsg3(ec)-His are large molecules and their conformation is apparently not stable enough to withstand regeneration procedures. Taking into consideration that not all anti-Dsg antibodies are of pathogenic relevance[4,97], it might be possible that some epitopes of Dsg1 and Dsg3 are of no importance concerning therapeutical application of adsorbers. Potentially, reduction of the size of the antigens to promote their stability will not influence binding of pathogenic autoantibodies. Shortening both desmogleins on genetic level was done. Since most epitopes of pemphigus autoantibodies are mapped to the N-terminal region of desmogleins (EC1 and EC2)[31,47,71,84,111,126,136], deletion of the C-terminal domains seemed a suitable approach to narrow down immunodominant regions of Dsg1 and Dsg3. To avoid exclusion of important epitopes on other extracellular domains, which might be present despite the published literature, I wanted to express each of the extracellular domains individually. However, the signal sequence and the propeptide, that reside N-terminally of EC1, are of high importance with respect to correct processing and localisation of Dsg1 and Dsg3. Producing extracellular domains alone might have resulted in unpredictable effects on protein expression and localisation, as was observed in C-cadherin deletion mutants[32]. To avoid this, individual extracellular domains were fused C-terminally to EC1, which harboures signal sequence and propeptide.

Table 4: Reactivity of Pemphigus sera with Dsg-deletion mutants

Dsg1	reactive sera	Dsg3	reactive sera
EC1-5	13/13	EC1-5	11/11
EC1-4	12/13	EC1-4	11/11
EC1-3	11/13	EC1-3	6/11
EC1-2	0/13	EC1-2	2/11
EC1	2/13	EC1	0/11
EC1+3	0/13	EC1+3	0/11
EC1+4	4/13	EC1+4	0/11
EC1+5	12/13	EC1+5	0/11
negative control	0/13	negative control	0/11

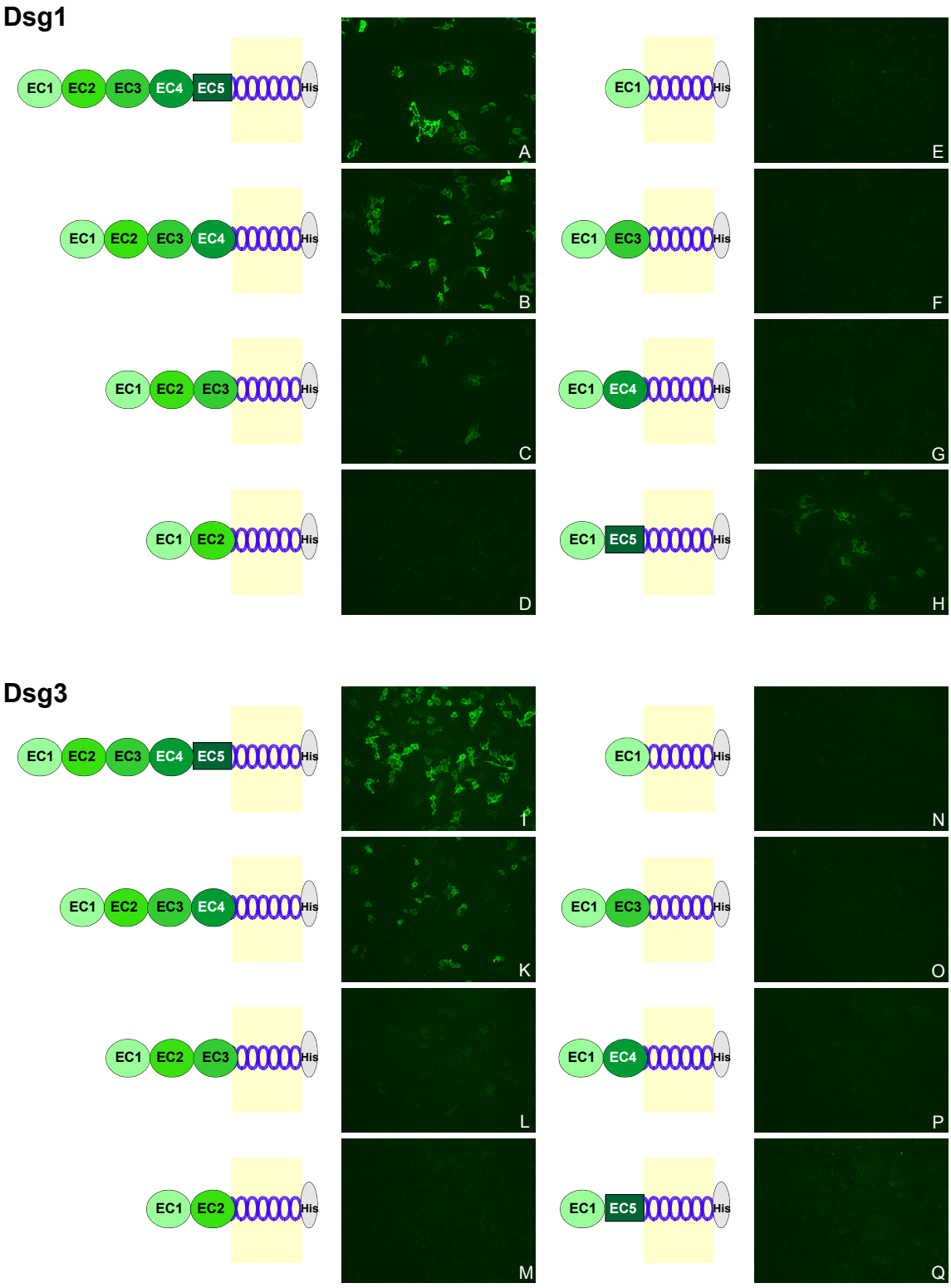

Figure 27: Immunofluorescence analysis of Dsg deletion mutants
HEK 293T cells were seeded onto glass slides and transfected with plasmids coding for different Dsg deletion proteins. Then the glass slides were submitted to BIOCHIP production as described in chapter 3.4. BIOCHIPs were incubated with anti-Dsg1 positive sera (A-H) or anti-Dsg3 positive sera (I-Q), respectively. A polyclonal goat anti-human IgG Cy2-conjugate was used as secondary antibody. A: Dsg1(ec)-TM; B: Dsg1_EC1-4_TM; C: Dsg1_EC1-3_TM; D: Dsg1_EC1-2_TM; E: Dsg1_EC1_TM; F: Dsg1_EC1+3_TM; G: Dsg1_EC1+4_TM; H: Dsg1_EC1+5_TM; I: Dsg3(ec)-TM; K: Dsg3_EC1-4_TM; L: Dsg3_EC1-3_TM; M: Dsg3_EC1-2_TM; N: Dsg3_EC1_TM; O: Dsg3_EC1+3_TM; P: Dsg3_EC1+4_TM; Q: Dsg3_EC1+5_TM.

A total of seven recombinant variants of each Dsg were cloned: EC1-4, EC1-3, EC1-2, EC1, EC1+3, EC1+4 and EC1+5. These proteins were expressed with the authentic transmembrane domains of Dsg1 and Dsg3, respectively, for use in immunofluorescence stud-

ies. This technique was chosen because it enables binding studies of proteins in their natural conformation and glycosylation without any artefacts caused by purification procedures. All Dsg deletion mutants were transiently expressed in HEK 293T cells. Expression of the proteins was confirmed with rabbit sera (data not shown). The transfected cells were incubated with pemphigus sera and stained for immunofluorescence. Figure 27 shows representative examples of observed autoantibody binding.

Dsg1(ec)-TM and Dsg3(ec)-TM represented the full length extracellular domains (EC1-5) of Dsg1 and Dsg3 and were used as positive controls. Indeed, all of the tested anti-Dsg1 positive sera reacted with Dsg1(ec)-TM, and all tested PV sera stained Dsg3(ec)-TM (summary of results is given in Table 4). Both desmogleins were able to bind pemphigus autoantibodies down to EC1-3 upon C-terminal deletion. When Dsg1_EC1-2 was expressed, there was no signal with the anti-Dsg1 positive sera and only 2 of 11 PV sera reacted with Dsg3_EC1-2. This was unexpected, because EC1 and EC2 are said to be the regions containing most epitopes of both desmogleins. Dsg1_EC1 bound IgG of two sera. Dsg1_EC1+3 was unreactive, whilst Dsg1_EC1+4 could be stained with 4 anti-Dsg1 positive sera and Dsg1_EC1+5 with 12 of 13 sera. The one serum that did not bind to Dsg1_EC1+5 had a very low titre to begin with and did hardly react with the positive control (Dsg1(ec)-TM). Dsg3_EC1 showed no binding of human antibodies, as well as Dsg3_EC1+3, Dsg3_EC1+4 and Dsg3_EC1+5.

The finding that Dsg1_EC1+5 bound anti-Dsg1 IgG of nearly all of the tested sera was probably due to correct folding of EC1 when expressed in fusion with EC5 (see Discussion). In the hope of covering additional epitopes on EC2, a protein consisting of Dsg1_EC1+2+5 was cloned and tested via immunofluorescence. Unfortunately, only 2 samples of a collective of 24 anti-Dsg1 positive pemphigus sera showed binding of this protein, although 16 sera of this collective reacted with Dsg1_EC1+5 (Table 5, Figure 28). In a similar approach, EC5 of Dsg1 was fused to EC1 of Dsg3 in order to get a correctly folded EC1 domain of Dsg3. But this protein was not reactive with any of the 18 PV sera tested (Table 5, Figure 29).

Table 5: reactivity of pemphigus sera with additional Dsg variants

Dsg1	reactive sera	Dsg3	reactive sera
EC1-5	24/24	EC1-5	18/18
EC1+5	16/24	Dsg3_EC1+Dsg1_EC5	0/18
EC1+2+5	2/24	negative control	0/18
negative control	0/24		

Taken together, there was one Dsg1 deletion construct able to bind anti-Dsg1 autoantibodies, namely Dsg1_EC1+5. In terms of Dsg3, no shortened protein reacted with pemphigus sera.

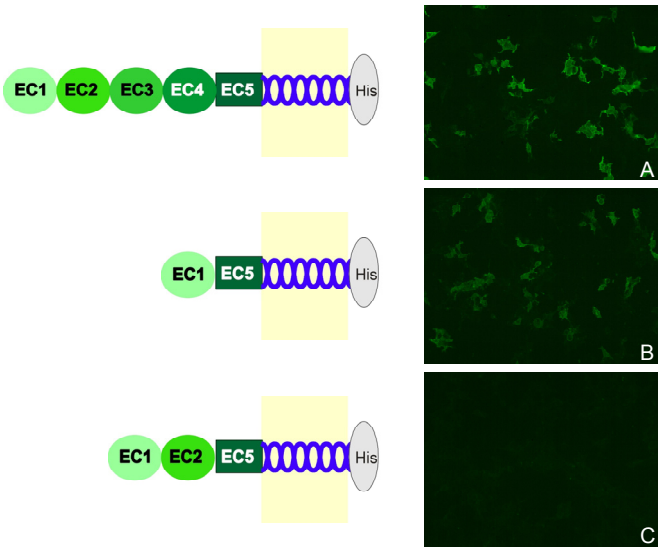

Figure 28: Dsg1_EC1+2+5 does not bind anti-Dsg1 IgG
HEK 293T cells were seeded onto glass slides and transfected with plasmids coding for different Dsg deletion proteins. Then the glass slides were submitted to BIOCHIP production as described in chapter 3.4. BIOCHIPs were incubated with anti-Dsg1 positive sera. A polyclonal goat anti-human IgG Cy2-conjugate was used as secondary antibody. A: Dsg1(ec)-TM; B: Dsg1_EC1+5_TM; C: Dsg1_EC1+2+5_TM.

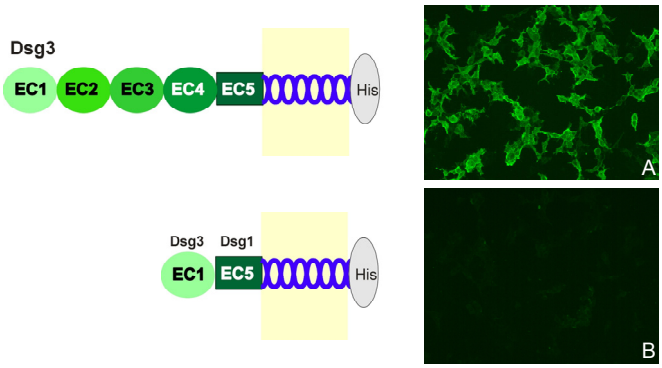

Figure 29: Dsg3_EC1+Dsg1_EC5 does not bind anti-Dsg3 IgG
HEK 293T cells were seeded onto glass slides and transfected with plasmids coding for different Dsg deletion proteins. Then the glass slides were submitted to BIOCHIP production as described in chapter 3.4. BIOCHIPs were incubated with anti-Dsg3 positive sera. A polyclonal goat anti-human IgG Cy2-conjugate was used as secondary antibody. A: Dsg3(ec)-TM; B: Dsg3_EC1+Dsg1_EC5_TM.

After discovering that Dsg1_EC1+5 was able to bind IgG of anti-Dsg1 positive sera, the next step was to create an adsorber based on this protein. To accomplish this, the transmembrane domain had to be removed from the C-terminus of the antigen and be replaced by a His-tag. This way, Dsg1_EC1+5 became soluble in aqueous buffers and was secreted into the cell culture supernatant by transfected HEK 293T cells. After purification via IMAC (see chapter 3.6), Dsg1_EC1+5_His was coupled to NHS-activated Sepharose and to AminoLink Plus agarose using the conditions established for Dsg1(ec)-His (see chapter 4.1.3).

Figure 30 shows the control Western blot for the coupling of the protein. After the immobilisation reaction to NHS-Sepharose, there was no antigen detectable anymore, indicating complete binding of the used antigen. Coupling to AminoLink agarose was not exhaustive, but the remaining amount of protein was small. Hence, both matrices bound

Dsg1_EC1+5-His very well. Probably, an even higher antigen load would have been possible, but for comparison with the full length extracellular domain of Dsg1, the same coupling amount was used for both proteins.

After coupling both Dsg1(ec)-His and Dsg1_EC1+5_His to NHS-Sepharose and AminoLink Plus agarose, the artificial PF pool was adsorbed on these matrices (Figure 31). Adsorption on Dsg1_EC1+5 led to less decrease of the anti-Dsg1 IgG titre than adsorption on Dsg1(ec)-His. 64 % and 62 % of the antibodies remained in the flow through, respectively, whereas Dsg1(ec)-His could lower the amount of anti-Dsg1 antibodies to 25 % and 14 %.

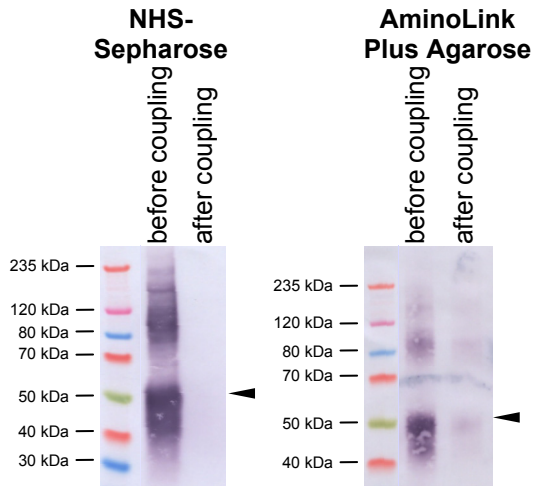

Figure 30: Coupling of Dsg1_EC1+5 onto both solid supports is highly efficient
Dsg1_EC1+5_His was immobilised on different preactivated solid supports with a density of 3 µg protein per µL NHS-Sepharose and 5 µg protein per µL AminoLink Plus agarose. Control samples were taken before and after coupling for Westernblot analysis. Detection of the recombinant protein was achieved with a monoclonal anti-His-tag antibody. The molecular weight standard is given on the left. Arrowheads show Dsg1_EC1+5_His.

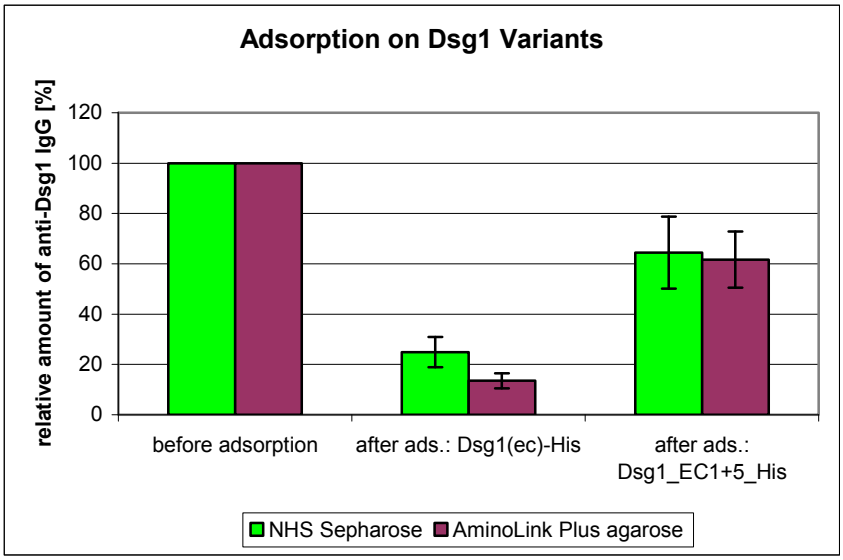

Figure 31: Dsg1(ec)-His is more efficient in binding anti-Dsg1 IgG than Dsg1_EC1+5
Dsg1(ec)-His and Dsg1_EC1+5_His were coupled to NHS-Sepharose with a density of 3 µg/µL solid support and to AminoLink agarose with a density of 5 µg/µL resin. The artificial PF sera pool was adsorbed on these matrices (n =3). After adsorption, the amount of anti-Dsg1 antibodies in the flow-through was measured by anti-Dsg1 ELISA and set in relation to the amount of anti-Dsg1 antibodies before adsorption (100 %). Displayed are mean values, indicated by error bars are standard deviations.

The difference in adsorption capacity is most likely caused by the fact that Dsg1_EC1+5 does not represent all epitopes present on the full length extracellular domain of Dsg1. Repeated adsorption of the sera pool on this protein did not result in further decrease of the amount of autoantibodies. Nevertheless, this reduction of the anti-Dsg1 titre would be sufficient if the antibodies not binding to Dsg1_EC1+5 were not pathogenic.

4.3 Pathogenicity of Pemphigus Sera

4.3.1 Acantholytical Effects of the Artificial PF Sera Pool

To determine the pathogenic potential of anti-Dsg1 antibodies, an *in vitro* assay based on fragmentation of HaCaT cells can be used[35]. Sera or antibodies of pemphigus foliaceus patients are incubated with confluent cells for several hours. Then the keratinocyte monolayer is dispatched from the culture vessel and subjected to mechanical stress. If the cells have been treated with pathogenic antibodies, the monolayer dissociates into multiple fragments, whereas incubation with non-pathogenic antibodies or sera only causes slight fragmentation.

The artificial PF sera pool was adsorbed on NHS-Sepharose and AminoLink agarose loaded with Dsg1(ec)-His or Dsg1_EC1+5_His as shown above. As a control, empty matrices were used. The flow through of individual adsorptions was then tested in the cell fragmentation assay (Figure 32).

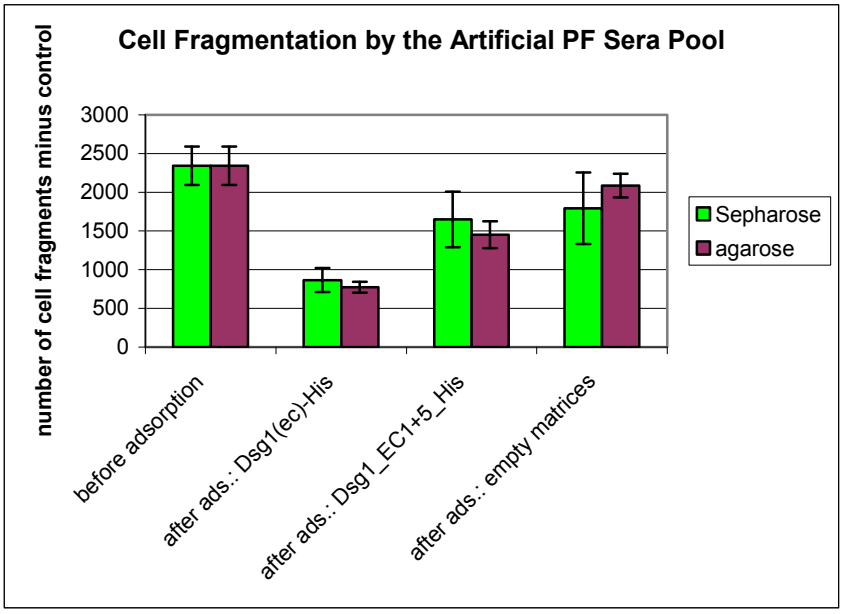

Figure 32: Adsorption on Dsg1 matrices decreases pathogenicity
The artificial PF sera pool was adsorbed on solid supports loaded with Dsg1(ec)-His, Dsg1_EC1+5_His or no protein (empty matrices). The flow through as well as the untreated sera pool were used for fragmentation of HaCaT cells (see chapter 3.10.1). The serum of a healthy blood donor served as negative control. Displayed are mean values of the numbers of cell fragments (n = 3). Indicated by error bars are standard deviations.

Adsorption of the sera pool on Dsg1(ec)-His significantly reduced the degree of cell fragmentation compared to untreated sera pool (Sepharose: $p < 0.05$; agarose: $p < 0.001$). This shows that adsorption of anti-Dsg1 IgG on the full length extracellular domain of Dsg1 decreases the pathogenicity of the antibody solution. In contrast to this, adsorption on blocked matrices caused no significant reduction of the number of cell fragments ($p > 0.05$). Processing with Dsg1_EC1+5 matrices reduced cell fragmentation, but this was only significant in case of Dsg1_EC1+5 agarose ($p < 0.01$). In comparison to the adsorption on Dsg1(ec)-His, the sera pool treated with Dsg1_EC1+5 Sepharose or agarose was more pathogenic.

Interpreting the results of this *in vitro* assay, it became clear that, though Dsg1_EC1+5 is able to bind pathogenic antibodies, there is also a portion of pathogenic IgG recognising epitopes outside EC1+5. Because of this, using this deletion variant of Dsg1 for therapeutical application was not considered any further.

The cell fragmentation assay permits quantification of pathogenicity, but has the disadvantage of high variation and bad reproducibility. In order to find another means of detecting pathogenic activity of anti-Dsg1 antibodies, two pathways involved in acantholysis were used as markers: phosphorylation of p38 MAPK and retraction of actin filaments.

To detect increased phosphorylation of p38 MAPK, HaCaT cells were analysed by Western blotting after incubation with human sera. Phosphorylation of p38 MAPK was increased after incubation with the PV sera pool, but also after incubation with a serum of a healthy blood donor (Figure 33), and is, therefore, unspecific.

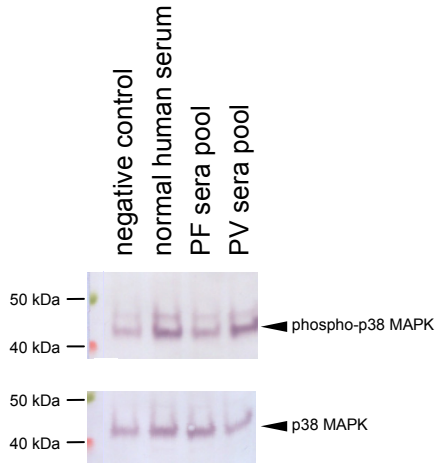

Figure 33: Phosphorylation of p38 MAPK in HaCaT cells upon incubation with human sera
HaCaT cells were incubated with normal human serum, the artificial PF sera pool or the artificial PV sera pool, respectively. Untreated cells served as negative control. After 30 min, cells were harvested and subjected to Westernblot analysis using an antibody specifically recognising phosphorylated p38 MAPK. The total amount of p38 MAPK was detected as loading control. The molecular weight standard is given on the left, arrowheads show phospho-p38 MAPK or p38 MAPK.

Reorganisation of actin filaments was visualised via immunofluorescence (Figure 34). In negative controls (Figure 34A and B), actin was located along the cell borders. The

same staining pattern was observed in HaCaT cells treated with the artificial PF sera pool. Only cells incubated with the artificial PV sera pool showed retracted actin fibres.

Phosphorylation of p38 MAPK and retraction of actin were not suitable to detect pathogenic effects of anti-Dsg1 IgG. Thus, no improved quality of information regarding pathogenic anti-Dsg1 autoantibodies could be achieved.

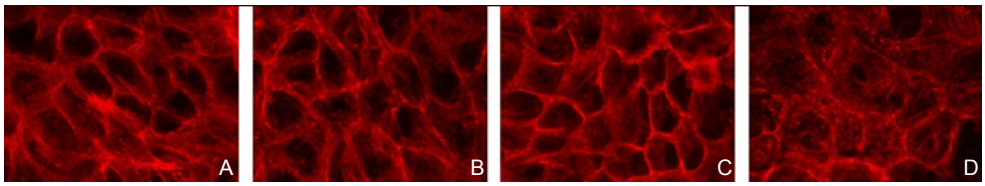

Figure 34: Actin reorganization in HaCaT cells as consequence of incubation with pathogenic sera
HaCaT cells were incubated with human sera for 24 h and stained with phalloidin-TRITC to detect actin fibres. A: negative control; B: normal human serum; C: artificial PF sera pool; D: artificial PV sera pool. A, B and C show normal distribution of actin along the cell membrane, whereas in D the membrane pattern is dissolved and actin is retracted into the cytoplasm.

4.3.2 Acantholytical Effects of the Artificial PV Sera Pool

The cell fragmentation assay can also be used to evaluate pathogenicity of anti-Dsg3 antibodies[61]. However, since this assay shows bad reproducibility, a different approach was chosen. HaCaT cells express much more Dsg3 than Dsg1, enough to visualise it via indirect immunofluorescence. Although Dsg1 can be detected in HaCaT cells under certain culture conditions[35], in these cases the cells do not form monolayers. They grow in kind of "stacks" [own observations], which is unsuitable for immunofluorescence studies.

Incubation of HaCaT cells with pathogenic anti-Dsg3 antibodies leads to clustering and degradation of Dsg3 that can be observed by immunofluorescence staining[29,34,132]. Based on this finding, an *in vitro* pathogenicity assay was developed[132]. This assay was modified for the requirements of this study.

HaCaT cells were incubated with serum samples and subsequently fixed and stained with a monoclonal anti-Dsg3 antibody (Figure 35). In untreated HaCaT cells, Dsg3 was homogenously distributed along the cell membrane in most cells, forming a netlike pattern with some extensions into the cells (Figure 35A). The same distribution of Dsg3 was observed in HaCaT cells pre-treated with normal human serum or with the artificial PF sera pool (Figure 35B and C). In contrast to this, incubation of the cells with the artificial PV sera pool led to an overall decrease of fluorescence intensity (Figure 35D). Dsg3 was hardly detectable in most cells. Still existing membrane staining was not homogenous anymore, but clustered with gaps between the individual fluorescing dots. These hallmarks of pathogenicity were not present when keratinocytes were treated with PV pool adsorbed on Dsg3 matrices (Figure 35E and F), indicating that pathogenic autoantibodies had been removed. After pre-treatment with artificial PV pool adsorbed on empty solid supports

(Figure 35G and H), Dsg3 staining of HaCaT cells did not show differences to the pattern observed after incubation with the unadsorbed sera pool.

These findings demonstrate that pathogenic anti-Dsg3 antibodies can be removed by adsorption on Dsg3 Sepharose and Dsg3 agarose, but not by adsorption on blocked matrices.

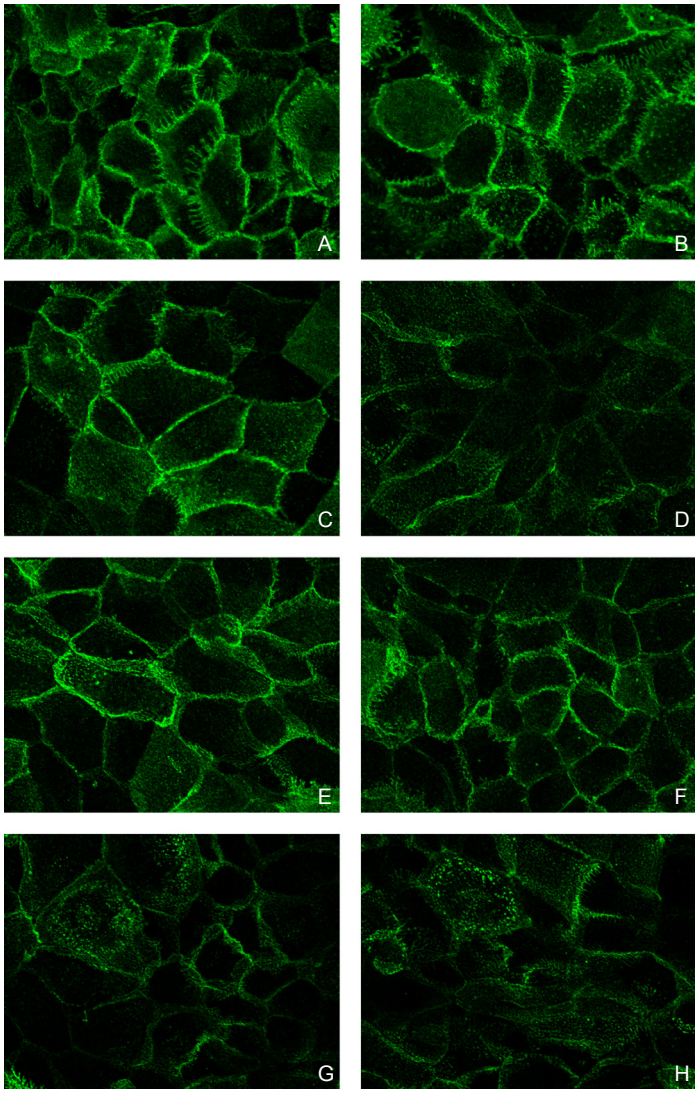

Figure 35: Reorganisation of Dsg3 in HaCaT cells
The artificial PV pool was adsorbed on different matrices. Then HaCaT cells were incubated with these samples for 48 h and subsequently fixed and stained with an anti-Dsg3 antibody. A: negative control; B: healthy blood donor; C: artificial PF pool; D: artificial PV pool before adsorption; E: artificial PV pool, adsorbed on Dsg3-Sepharose; F: artificial PV pool, adsorbed on Dsg3-agarose; G: artificial PV pool, adsorbed on blocked NHS-Sepharose; H: artificial PV pool, adsorbed on blocked AminoLink Plus agarose.

4.3.3 Acantholytical Effects of IgG4 Depleted PV Sera Pool

It could be shown that Dsg adsorbers are capable of specifically binding anti-Dsg autoantibodies and that adsorption reduces pathogenicity of pemphigus sera. However, as has been demonstrated above, regeneration of Dsg adsorbers is associated with problems most likely resulting from instability of the antigens. To avoid high production costs, more stable proteins are needed. Since no Dsg deletion mutant could be found that was able to bind all pathogenic autoantibodies, removal of anti-Dsg antibodies via their Fc-part was considered.

Anti-Dsg IgG in pemphigus patients belong mainly to the subclasses IgG1 and IgG4[22,41,51,116]. Evidence is accumulating that IgG4 directed against desmogleins are of more pathogenic relevance than IgG1[15,22,41,41,50,116,129]. In addition, IgG4 is the least abundant of the four IgG subclasses[87]. Therefore, removing IgG4 from pemphigus sera seemed a rational therapeutical approach for these patients.

To accomplish this, a monoclonal antibody directed against the Fc-part of human IgG4 was coupled to NHS-Sepharose. 4 µg of the antibody per µL Sepharose could be coupled with high efficiency, since the amount of protein in the control sample of the reaction was decreased to almost the detection level (Figure 36).

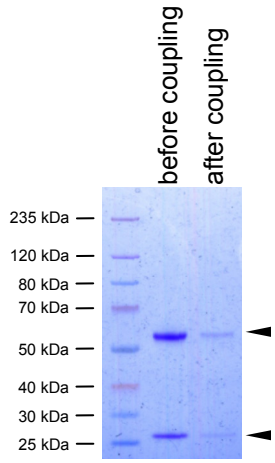

Figure 36: Coupling of an anti-human IgG4 antibody to NHS-activated Sepharose
A monoclonal antibody directed against the Fc-part of human IgG4 was coupled to NHS-activated Sepharose with a density of 4 µg protein per µL solid support. Before and after coupling, samples were taken and analysed by Coomassie staining. The molecular weight standard is given on the left, arrowheads indicate the positions of heavy and light chain of the antibody. Protein concentration is significantly decreased after the coupling procedure.

After successful coupling, a small volume of the artificial PV sera pool was adsorbed on anti-IgG4 Sepharose to remove all IgG4 antibodies. The amount of IgG directed against Dsg3 (whole IgG and IgG4) was measured by ELISA (Figure 37). Anti-Dsg3 IgG4 could be removed almost completely, whereas 27 % of the overall IgG directed against Dsg3 still resided in the sera pool. In conclusion, this means that 73 % of the anti-Dsg3 IgG in this sera pool belong to the IgG4 subclass.

To determine the pathogenicity of the adsorbed sera pool, the immunofluorescence assay already used in chapter 4.3.2 was carried out (Figure 38). Negative controls (Figure 38A and B) show the typical membrane staining of Dsg3. Upon pre-incubation with untreated PV sera pool (Figure 38C), Dsg3 became clustered and immunofluorescence intensity was considerably weaker compared to the controls. When the PV pool was adsorbed on anti-IgG4 Sepharose, it still caused gaps in the membrane staining and low fluorescence intensity (Figure 38D). This indicates that pathogenicity of the sera pool could not be abolished despite the almost complete removal of anti-Dsg3 IgG4. Eluted anti-Dsg3 IgG4 led to an immunofluorescence pattern exhibiting all features of pathogenicity: decreased fluo-

rescence intensity of Dsg3, clustering and gaps along the cell membrane (Figure 38E). This clearly demonstrates that anti-Dsg3 IgG4 are pathogenic, but not the only cause of Dsg3 degradation and acantholysis.

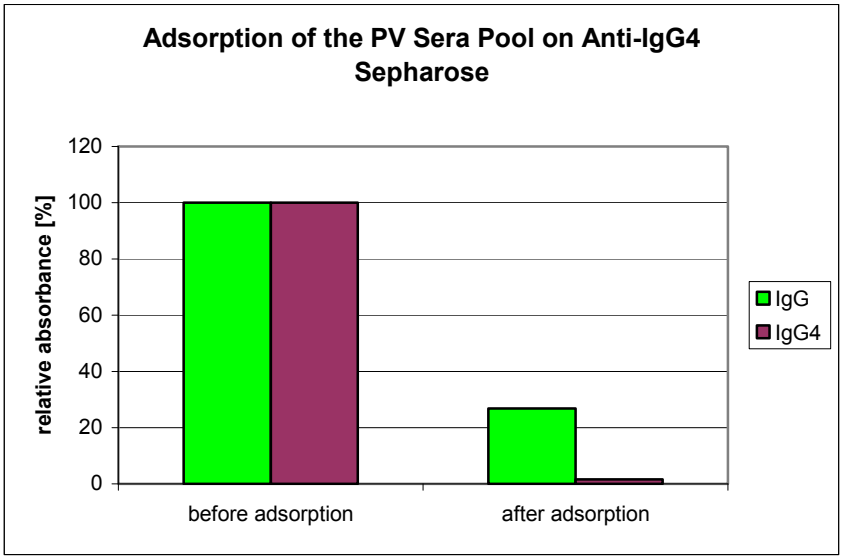

Figure 37: IgG4 can be removed from the artificial PV sera pool
The artificial PV sera pool was adsorbed on an anti-human IgG4 antibody coupled to NHS-activated Sepharose (50 µL serum per 25 µL matrix, n = 1). The flow-through was analysed by anti-Dsg3 ELISA. As secondary antibody, a POD-conjugate directed against the Fc-part of all human IgG was used on the one hand, on the other hand a conjugate specific for human IgG4 alone was utilised. The absorbance of the samples after adsorption was set in relation to the absorbance of the samples taken before adsorption (100 %). After adsorption, there was hardly any anti-Dsg3 specific IgG4 detectable.

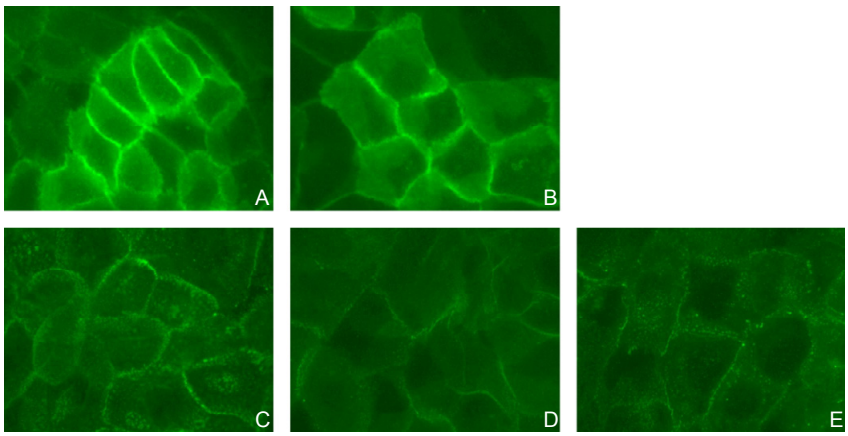

Figure 38: Pathogenicity of the PV sera pool after removal of IgG4
The artificial PV pool was adsorbed on anti-IgG4 Sepharose, incubated with HaCaT cells and stained with an anti-Dsg3 antibody. A: negative control; B: healthy blood donor; C: artificial PV pool before adsorption; D: artificial PV pool after adsorption; E: eluted antibodies.

Summing up these data, anti-IgG4 adsorbers would presumably be able to remove most of the pathogenic autoantibodies of PV patients, but not all of them. Thus, the idea of using anti-IgG4 antibodies was not pursued.

4.4 Extraction of anti-Dsg IgG from Individual Pemphigus Sera

After every attempt to use more stable proteins for the development of immunoadsorbers than Dsg1(ec)-His and Dsg3(ec)-His had failed, it was decided to utilise these recombi-

nant desmoglein variants despite their disadvantages. After all, Dsg1(ec)-His and Dsg3(ec)-His had proven to be both very effective and specific with regard to the binding of anti-Dsg antibodies in the artificial sera pools.

Since most experiments up to this point had been carried out with artificial sera pools, it was very interesting to see if these results would be transferable to individual patients. To analyse this, ten sera of PF and PV patients each had been collected and were adsorbed on the respective Dsg matrices.

The results of the ELISA analysis of the adsorbed PF sera are displayed in Figure 39. From those sera with a low titre (PF3 and PF7), anti-Dsg1 IgG could be removed nearly completely with both Dsg1 Sepharose and Dsg1 agarose. With all other sera, Dsg1 agarose was clearly more efficient than Dsg1 Sepharose. Residual anti-Dsg1 titres ranged from 14 % to 90 % after adsorption on Dsg1 Sepharose, whereas Dsg1 agarose was able to decrease anti-Dsg1 IgG to < 1 % to 40 %. It was noticed that the residual portion of anti-Dsg1 antibodies after adsorption did not necessarily correlate with the anti-Dsg1 titre before adsorption, as one would have expected. For example, in PF9 there were less autoantibodies left after adsorption on Dsg1 Sepharose (46 %) than in PF1 (57 %) or PF10 (61 %), although the starting titre was with 315 RU/mL considerably higher than that of PF1 and PF10 (195 and 265 RU/mL).

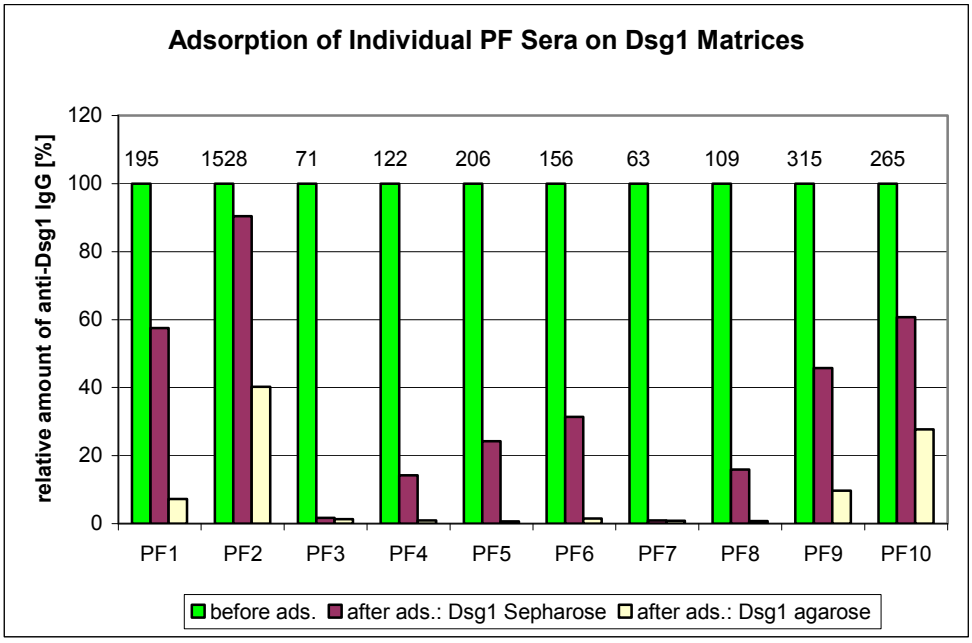

Figure 39: Differences in the adsorption of individual PF sera on Dsg1 matrices
Ten PF sera were adsorbed on Dsg1 Sepharose and Dsg1 agarose (n = 1 for each serum). Before and after adsorption, the sera were analysed by anti-Dsg1 ELISA. The amount of anti-Dsg1 IgG after adsorption was set in relation to the amount before adsorption (100 %). Numbers above the green columns represent the anti-Dsg1 titre in RU/mL before adsorption.

Similar effects were observed during the adsorption of PV sera, although they were less prominent (Figure 40). The serum with the highest anti-Dsg3 titre was PV1 with 602 RU/mL, but after adsorption, the residual amount of anti-Dsg3 antibodies was less

than 5 % independent of the used solid support. There were some sera with lower starting titre than PV1, but more residual anti-Dsg3 IgG after adsorption: PV5 (2/10 %), PV8 (10/19 %) and PV10 (2/16 %).

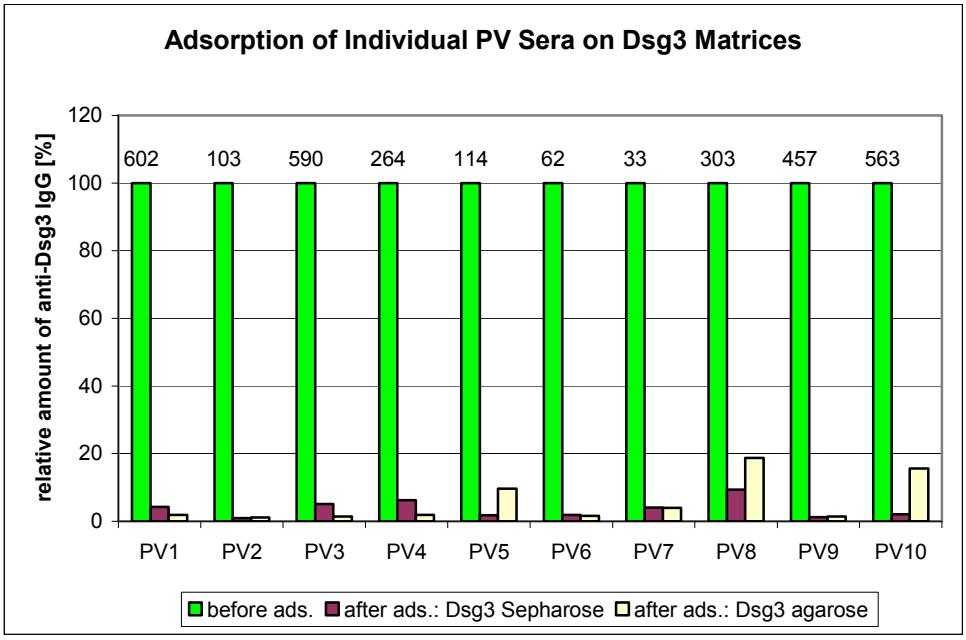

Figure 40: Differences in the adsorption of individual PV sera on Dsg3 matrices
Ten PV sera were adsorbed on Dsg3 Sepharose and Dsg3 agarose (n = 1 for each serum). Before and after adsorption, the sera were analysed by anti-Dsg3 ELISA. The amount of anti-Dsg3 IgG after adsorption was set in relation to the amount before adsorption (100 %). Numbers over the green columns represent the anti-Dsg3 titre in RU/mL before adsorption.

Both Dsg3 adsorbers were able to decrease the residual anti-Dsg3 titre to less than 20 % in all sera. There were differences between adsorption efficiency on Dsg3 Sepharose and Dsg3 agarose, but no matrix was better in all cases. In fact, half of these sera could be adsorbed more efficiently on Dsg3 Sepharose, whereas for the other half Dsg3 agarose would be the more appropriate matrix.

Although the performance of Dsg1 and Dsg3 adsorbers differed with respect to individual pemphigus sera, there was no serum that was “resistant” to antibody removal. It can be concluded that immunoadsorption worked with all tested samples. In some patients, however, it would be necessary to use a higher amount of Dsg matrix to remove all anti-Dsg antibodies. Concerning adsorption on Dsg1, AminoLink Plus agarose seems much better suited as solid support than NHS-activated Sepharose.

5. Discussion

5.1 Properties of Recombinant Desmogleins

The extracellular domains of Dsg1 and Dsg3 are the primary targets of autoantibodies in pemphigus diseases. In order to bind these antibodies, recombinant desmogleins representing the complete extracellular domains were immobilised onto six different solid supports and tested with respect to their adsorption capacity (chapters 4.1.1 and 4.1.2). Anti-Dsg3 IgG could be adsorbed efficiently with two of the six tested solid supports, namely NHS-activated Sepharose and AminoLink Plus agarose. In contrast to this, in preliminary experiments only NHS-Sepharose was capable of binding anti-Dsg1 IgG to a similar extent. The cause for the differences in the behaviour of Dsg1 and Dsg3 matrices in adsorption is unknown, but it is likely that the conformation of Dsg1(ec)-His is more sensitive to coupling conditions (e.g. pH) than that of Dsg3(ec)-His. Loss of natural conformation, in turn, most likely leads to less binding of autoantibodies. By quintupling the buffer concentration in the AminoLink coupling solution, it was possible to stabilise the pH and immobilise Dsg1(ec)-His in a way that binding of anti-Dsg1 IgG was as efficient as on Dsg1 Sepharose.

Dsg Sepharose and Dsg agarose were optimised regarding the ratio of coupled antigen and bound IgG (chapter 4.1.3). The higher the antigen load on both solid supports, the lower were residual amounts of anti-Dsg antibodies, although immobilisation of Dsg1(ec)-His and Dsg3(ec)-His was incomplete at high concentrations. This might be due to the fact that nucleophilic substitutions are basically equilibrium reactions. They are pushed towards the formation of products when the amount of educts is raised. Therefore, one can assume that the actual protein load of the solid supports can be influenced even after apparent saturation of the matrices is reached.

Although it has not been described in the literature, it became apparent in the course of this work that the extracellular domain of Dsg1 is considerably more instable than that of Dsg3. During elution of anti-Dsg1 IgG from the adsorbers, the antigen was damaged in a way that led to less binding of autoantibodies (chapter 4.1.5). Although this was also observed with respect to Dsg3 matrices, the decrease in adsorption capacity was considerably smaller in these cases. Since neither separation of the antigens from the solid phases nor incomplete elution of autoantibodies are the cause for the loss of adsorption performance, damage of conformational epitopes is the most likely explanation for the lacking stability of Dsg (especially Dsg1) adsorbers.

Instability of Dsg1(ec)-His was not only observed during adsorption and regeneration studies, but also in everyday procedures. For example, Dsg1(ec)-His does not endure

freeze-thawing cycles in simple aqueous buffers. At least 20 % of glycerol have to be added to the storage buffer to retain antibody binding activity of the protein. Dsg3(ec)-His does not need such additives.

The cause for this difference of the two antigens might lie in their EC5 domains. EC5 of Dsg1 is an atypically short membrane proximal extracellular domain, whereas EC5 of Dsg3 shows limited sequence homology to cadherin domains[90]. Up to now, no crystal structure of Dsg1 or Dsg3 has been described, but from immunofluorescence studies of Dsg deletion mutants (see chapter 4.2), it can be concluded that EC5 influences folding of cadherin domains and that the effects of EC5 on the conformation differ between Dsg1 and Dsg3. This is based upon the following findings:

I. All tested Dsg1 deletion mutants contained EC1, but only Dsg1_EC1-5, EC1-4 and EC1+5 showed reactivity with nearly all tested PF sera, indicating that folding of EC1 is influenced by the C-terminus of the protein.
II. Dsg1_EC1+5 bound PF antibodies in considerably more cases than Dsg1_EC1+2+5. Also, Dsg1_EC1-4 showed antibody binding in most samples. This makes the presence of relevant epitopes on EC5 unlikely.
III. In contrast to Dsg1_EC1+5, Dsg3_EC1+5 did not react with patients' sera. Since the EC1 domains of both desmogleins share 73 % amino acid sequence identity, this difference is most likely caused by the EC5 domains.

In previous reports, it was already assumed that the three-dimensional structure of the extracellular domains of Dsg1 and Dsg3 cannot be achieved in recombinant C-terminally truncated mutant proteins[47,111]. Although Müller et al. performed epitope mapping studies with single EC domains of Dsg3, binding activity of their protein variants to PV sera did not change in ELISA studies even after denaturing the proteins[85]. This indicates that the recognised epitopes were not conformational to begin with and that acquiring correctly folded EC domains of Dsg3 was not successful by expressing single domains alone.

Importance of the membrane proximal extracellular domains is underlined by another study, in which the authors investigated dimerisation and adhesion of C-cadherin mutants. They observed less binding of C-cadherin EC1-2 compared to EC1-5 in bead aggregation assays, although EC4 and EC5 did not seem to take part in homophilic C-cadherin interactions[32]. The authors concluded that EC1 and EC2 are not sufficient for effective aggregation activity, but it might as well be that these two N-terminal domains were not correctly folded due to the missing C-terminus. These facts lead to the conclusion that expression of all EC domains of Dsg1 and Dsg3 is necessary to represent all epitopes relevant for autoantibody binding.

Although it would be sufficient to remove only pathogenic antibodies, which might be achieved by just a few epitopes, it has been reported that several apathogenic mouse anti-Dsg3 antibodies can cause dissociation of keratinocytes when used in combination[68]. Therefore, displaying pathogenically irrelevant epitopes in addition to pathogenic ones on an adsorber seems to be the safest approach for therapeutical applications. In addition, several attempts to represent Dsg epitopes that have been carried out together with our cooperation partners, have failed. This includes a peptide array derived from the Dsg3 amino acid sequence[1,42] and homology modelling of Dsg3 in order to map immunodominant regions [O. Kreuzer, peptides&elephants, personal communication]. Peptide phage display with a random peptide database was also tried. This method can be used to enrich and sequence peptides or proteins specifically binding to a certain ligand[59]. M13 phages presenting cyclic 7mer peptides were panned against purified anti-Dsg antibodies in order to find amino acid sequences displaying the surface of important Dsg epitopes. Unfortunately, it was not possible to enrich phages carrying such mimotopes in this approach, probably due to the multitude of epitopes recognised by anti-Dsg antibodies.

The best expression systems to represent all epitopes of a human protein are human cells. In this study, HEK 293T cells were used because they can easily be transfected. But producing proteins in these cells has some major drawbacks. First, the cell culture medium is expensive and not suitable for production of certified medical devices, since it contains FBS. Second, protein yields are quite low compared to other expression systems. Especially when keeping in mind that Dsg adsorbers might have to be developed as single use devices, high amounts of recombinant desmogleins will be needed. Therefore, one has to consider the use of a different expression system. Bacteria or yeast are inappropriate, because they are not able to reproduce the authentic conformation and glycosylation of mammalian proteins. A frequently used expression system in Dsg research is the baculovirus system. Insect cells are infected with recombinant baculoviruses carrying the coding sequence of Dsg1 or Dsg3. This allows antigen production in high amounts and with low expenses[86]. However, posttranslational alterations of the proteins can only be done to a certain extent. When expression of exogenous antigens is too high, the endogenous machinery for posttranslational modifications is unable to manage processing all molecules[86]. This can lead to incomplete cleavage of the propeptide in case of desmogleins[112]. This might be disadvantageous regarding the use of those proteins in therapeutic adsorptions, since it has been shown that cleavage of the propeptides from desmogleins results in increased binding of pathogenic antibodies from both mice and humans[112,136]. This problem can be overcome by coexpression of Dsg1 and Dsg3 with furin, the protease that

cleaves desmogleins from their propeptides. Experiments to switch desmoglein production to such a baculovirus system are currently under way.

5.2 Specificity of Desmoglein Adsorbers

Adsorption of anti-Dsg matrices proved to be highly specific, since the amount of pemphigus unrelated IgG was not influenced (chapter 4.1.4). Measurement of total IgG levels also indicated that the percentage of anti-Dsg antibodies is very low compared to the whole of IgG. To determine the exact ratio between anti-Dsg and non-anti-Dsg IgG, however, different tests would have to be conducted. Adsorption on Dsg matrices are not the appropriate way to address this matter, because it cannot be excluded that hydrophobic antibodies (anti-Dsg as well as non-anti-Dsg) stick to the surface of reaction tubes or the carbohydrate supports.

Every immunoadsorption device used at present binds either all immunoglobulins or a whole Ig subclass. Although this has the advantage of utilising one device for a variety of applications (e.g. rheumatoid arthritis, dilative cardiomyopathy, antibody mediated rejection of grafts, Goodpasture syndrome, systemic lupus erythematosus, bullous autoimmune dermatoses[26,104,140]), antibodies necessary for immune defence are removed in addition to those that cause diseases. Patients have to be supplied with intravenous IgG subsequent to immunoadsorption to avoid severe infections in some cases[43]. To circumvent these side effects, one focus in the development of Dsg matrices was maximum specificity. The goal of adsorbing only anti-Dsg antibodies without influencing pemphigus irrelevant IgG was reached. With this, patients will have to suffer less side effects.

Often, a feedback-induced rebound of autoantibody synthesis occurs in pemphigus patients treated with unspecific immunoadsorption due to the decreased levels of total IgG[13,43]. This was not observed in patients with dilative cardiomyopathy who underwent specific immunoadsorption for five consecutive days to remove antibodies directed against the β_1-adrenergic receptor. After treatment, the level of anti-β_1-adrenergic antibodies was decreased to approximately 20 % and did not rise again over a follow-up period of 12 month, during which no further immunoadsorption was administered[104]. Hopefully, anti-Dsg IgG will behave similar.

5.3 Pathogenicity of Adsorbed Pemphigus Sera

Pathogenicity of the artificial sera pools in *in vitro* assays was significantly reduced upon adsorption on Dsg matrices (chapter 4.3). Thereby, adsorbed sera were still more pathogenic than control sera. In the cell fragmentation assay, the number of cell fragments could not be decreased to control levels, and in case of Dsg3 adsorbers, individual cells showed small gaps in the Dsg3 immunofluorescence pattern. This was most likely caused

by the fact that the sera pools still contained about 20 % of anti-Dsg antibodies. It can be assumed that complete removal of pemphigus autoantibodies will completely abolish pathogenic effects in these assays. Experiments done at the Department of Dermatology and Venerology showed that individual pemphigus sera completely depleted of anti-Dsg IgG by use of the adsorbers at hand did not cause cell fragmentation or Dsg3 degradation anymore [J. Dworschak, personal communication].

Concerning anti-Dsg3 antibodies, the established immunofluorescence assay is meaningful and definite. Unfortunately, this assay cannot be transferred to anti-Dsg1 antibodies because of the low expression levels of Dsg1 in cultured keratinocytes. In order to avoid the imprecision implicated with the cell fragmentation assay, several attempts were made to develop an alternative pathogenicity assay. By detection of phosphorylation of p38 MAPK, no correlation between incubation with the artificial pemphigus sera pools and increased phosphorylation of p38 MAPK could be observed. Increased amounts of phospho-p38 MAPK have been described upon incubation with pemphigus sera in primary keratinocytes and in mice[17,19,20]. Also, extracts of perilesional skin of pemphigus patients showed this increased phosphorylation[17]. In all cases, normal keratinocytes are involved in the process of acantholysis. HaCaT cells, however, are an immortalised cell line showing some differences in differentiation and proliferation compared to primary keratinocytes[39]. It is possible that some pathways activated in primary cells upon contact with anti-Dsg IgG do not exist in HaCaT cells or are used to a lesser extent. Although in another study elevated levels of phospho-p38 MAPK were detected in HaCaT cells, too[119], the effect was much less prominent than in the previous works with primary cells. In addition, lower pathogenicity of the pemphigus sera used compared to those in the published literature might have prevented successful pursuit of this pathway in my work.

In a second approach, reorganisation of the actin skeleton upon incubation of pemphigus sera was used as criterion[131]. Unfortunately, retraction of actin fibres could only be observed after incubation with PV sera. PF sera had no effect on the distribution of actin filaments, probably due to the low expression rate of Dsg1 in HaCaT cells.

Low levels of endogenous Dsg1 in this cell line have repeatedly been a hindrance in research. For dissection of the effects of anti-Dsg1 antibodies *in vitro*, a stable, keratinocyte derived cell line is needed. For example, one could use lentiviral or adenoviral transduction to introduce Dsg1 under the control of a more active promoter into HaCaT cells. Preferentially, a simultaneous knock down of Dsg3 should be done to prevent compensation of the two proteins[76,114]. A HaCaT derived cell line expressing only little Dsg3, but instead Dsg1 at normal Dsg3 levels, could be a powerful tool in pemphigus research. So far, no such attempts have been done. The keratinocyte dissociation assay introduced by Ishii et al.[61],

therefore, remains the only established *in vitro* assay to examine the pathogenicity of anti-Dsg1 antibodies.

An alternative to *in vitro* pathogenicity assays is subcutaneous injection of pemphigus sera or antibodies into neonatal mice[5,8]. The mice develop blisters if injected antibodies are pathogenic. It is intended to test pemphigus sera adsorbed on Dsg matrices in this mouse model in cooperation with the Institute of Dermatology and Venerology at the University of Lübeck in the near future.

These *in vivo* studies will also be necessary to determine whether epitopes destroyed during regeneration procedures of the Dsg matrices bind pathogenic antibodies. IgG binding to conformational epitopes are considered to be related to acantholysis[52,97]. However, even if epitopes of pathogenic relevance depend on the conformation of the desmogleins, they might not necessarily be sensitive to acidic elution of antibodies. Examination of sera adsorbed on regenerated Dsg matrices will show if IgG not recognised anymore by Dsg1(ec)-His or Dsg3(ec)-His are capable of inducing blisters. If they are not, it would still be possible to develop Dsg adsorbers as multiple use devices.

As substitute for the instable full length extracellular domains of Dsg1 and Dsg3, an anti-human IgG4 antibody was coupled to NHS-activated Sepharose. This was promising because anti-Dsg3 antibodies of the IgG4 subclass have been reported to predominate in patients with active PV[15,22]. Furthermore, anti-Dsg1 antibodies in PV belong to the IgG4 subclass[41] and in endemic PF, an increase of the amount of anti-Dsg1 IgG4 is associated with the onset of disease[129]. Pemphigus autoantibodies mediate their pathogenic effects independently of their Fc portions[44,62,79,97], but IgG1 and IgG4 antibodies seem to differ with respect to their recognised epitopes[51,116]. Unfortunately, removing IgG4 from the artificial PV sera pool was not sufficient to prevent Dsg3 degradation in the *in vitro* assay. Although most anti-Dsg3 antibodies could be removed with the anti-IgG4 adsorber, the remaining IgG were still pathogenic. This clearly shows that other IgG apart from IgG4 are capable of inducing acantholysis. Though in part contradictory to the findings described above, this is in line with the observation that anti-Dsg1 antibodies of the IgG1 subclass can induce experimental PF[52]. For this reason, adsorption of IgG4 from the artificial PF sera pool was not done.

5.4 Differences between Adsorptions of Individual Sera

When sera of individual pemphigus patients were adsorbed on matrices loaded with Dsg1(ec)-His or Dsg3(ec)-His, all of these sera showed a clear reduction of the amount of anti-Dsg antibodies (chapter 4.4). This suggests that specific anti-Dsg immunoadsorption would be applicable to all PF and PV patients. From sera with a low starting amount of anti-Dsg antibodies (< 100 RU/ml), these IgG could be removed nearly completely. Con-

cerning sera with median and high titres of autoantibodies, the residual quantity of anti-Dsg IgG did not necessarily correlate with the titre before adsorption. One would expect the anti-Dsg titre after adsorption to be the higher, the more anti-Dsg IgG were present before adsorption. This was indeed the case in some sera, but there were also samples with moderate anti-Dsg starting titres and comparably high residual amount of anti-Dsg IgG and vice versa. The reason for this might lie in different compositions of the antibodies with respect to the epitopes they recognise. IgG are large molecules with a molecular weight of approximately 144 kDa. Compared to this, Dsg1(ec)-His and Dsg3(ec)-His are relatively small (≈ 80 kDa). If epitopes recognised by one serum are clustered, for example, on the N-terminus of the antigen, binding of one antibody might prevent adsorption of other IgG due to steric hindrance. Spreading of recognised epitopes throughout the whole extracellular domains of desmogleins, on the other hand, enables more IgG molecules per Dsg molecule to bind. Hence, more anti-Dsg antibodies can be removed with one adsorption step. Steric hindrance could be limited by site-specific (C-terminal) immobilisation of desmogleins onto the solid supports and additional use of linkers[30]. This would be a challenging task in terms of desmogleins, because several chemical reactions of both solid supports and antigens would have to be carried out without damaging the conformation of the proteins.

Interestingly, with some PV sera, adsorption was more efficient on Dsg3 agarose, whereas other PV sera could be better adsorbed on Dsg3 Sepharose. Derived from the assumption that adsorption efficiency is related to the epitope recognition profile of anti-Dsg IgG, one could conclude that distinct epitopes are accessible upon coupling to the two matrices. This might be a result of different pore sizes or densities of the reactive groups of the solid supports. Unfortunately, this will remain an assumption, since Fisher Scientific was not willing to release information regarding the structure and group density of AminoLink Plus agarose. It might also be possible that the ligands have a different orientation on the matrix due to distinct salt concentrations and pH values during coupling.

In terms of adsorption of PF sera, it was observed that Dsg1 agarose always performed better than Dsg1 Sepharose. Both activated solid supports react with primary amino groups, but conditions of coupling, washing and blocking are different. Washing of Dsg1 matrices in particular might contribute to the different adsorption efficiency. Dsg1 agarose is washed with a high salt buffer, whereas for Dsg1 Sepharose an acidic acetate buffer (pH 4) is used. Since Dsg1(ec)-His is much more sensitive to acidic conditions than Dsg3(ec)-His, washing of Dsg1 Sepharose with a pH 4 buffer might cause lack of antibody binding compared to Dsg1 agarose. This effect is not present in Dsg3 matrices, probably due to higher stability of Dsg3(ec)-His.

Following this argumentation, using AminoLink Plus agarose for the development of Dsg1 adsorbers seems rational. However, NHS-activated Sepharose has some advantages. The coupling procedure is very fast and does not require toxic reagents, which have to be removed thoroughly before application on humans. Furthermore, the protein load necessary for efficient adsorption is lower than that of AminoLink Plus agarose, which would make an adsorption device considerably cheaper and easier to produce.

A big disadvantage of NHS-Sepharose is the fact that this solid support cannot be sterilised by autoclaving previous to coupling procedures. The NHS group hydrolyses from the matrix when exposed to high temperatures and water. Autoclaving Sepharose with coupled proteins is possible, but Dsg1(ec)-His and Dsg3(ec)-His did not survive this procedure in preliminary experiments [data not shown]. This leaves irradiation as only means to sterilise Dsg Sepharose.

AminoLink Plus agarose itself cannot be autoclaved, too, but there are other aldehyde agarose resins available that are stable enough for these conditions (e.g. Actigel ALD by Sterogene Bioseparations). By using such a solid support, it would be possible to produce a sterile adsorption device when protein expression, purification and coupling are done in an aseptic environment. The premise for this is that autoclavable aldehyde agarose resins are as efficient as AminoLink Plus agarose. Experiments concerning this matter are already in progress.

5.5 Future Aspects

Apart from PF and PV, there are other pemphigus diseases during which antibodies directed against Dsg1 and/or Dsg3 occur, e.g. PNP, pemphigus herpetiformis, IgA pemphigus and drug induced pemphigus[80,115,123,138]. Out of this subset of pemphigus variants, especially PNP might be treated with Dsg adsorbers because of its severe pathogenesis and high mortality[110]. However, anti-Dsg antibodies are only detectable in a subgroup of patients. Further autoantibodies with pathogenic relevance seem to exist in these pemphigus variants.

According to the coupling of Dsg1(ec)-His and Dsg3(ec)-His, various autoantigens may be immobilised onto activated solid supports in order to create specific adsorption devices. These might be used to catch additional autoreactive antibodies in pemphigus diseases (e.g. anti-desmocollin 3 IgG[78,103]) or to treat different autoimmune disorders. There are a number of illnesses for which one or few main autoantigens have been described. Although autoantibodies are not in all cases known as sole cause for clinical symptoms, in some diseases the use of a specific immunoadsorption device would be promising. Bullous pemphigoid[40] (adsorber is already in development at EUROIMMUN), epidermolysis bullosa acquisita[74], dilative cardiomyopathy[104,128], rheumatoid arthritis[69] and myasthenia gra-

vis[23,36] are among them. Dsg adsorbers may serve as models and outrider projects for the creation of other specific immunoadsorbers.

With the current status of development of Dsg matrices, ten volumes of serum or plasma can be adsorbed on one volume of solid phase. At least 2 L of patient plasma are processed during one apheresis session. Hence, a minimum of 200 mL per Dsg adsorber will be necessary, assuming that regeneration of the columns will not be applicable. Depending on the solid phase, 0.6 to 1 g of protein will be needed per column. Transfer of the current batch procedures to chromatography will hopefully increase the volume of serum that can be adsorbed on Dsg matrices, thus reducing the amount of protein needed per treatment to a feasible value.

Using Dsg matrices for therapy of PF and PV patients has very good prospects, since they are specifically directed against the sole cause of blister formation. Influences on other parts of the immune system are not to be expected. This is an outstanding advantage compared to the current pemphigus therapy. Of course, effectiveness of the adsorption on Dsg matrices in human pemphigus patients still remains to be shown. From the *in vitro* assays, however, it can be assumed that specific immunoadsorption in PF or PV will lead to clinical improvement of patients that are otherwise therapy resistant. If the benefits of specific immunoadsorption have been demonstrated, it might even become a first line therapy, sparing the patients systemic immunosuppression. Frequency of apheresis sessions will depend on the time needed for reproduction of new anti-Dsg antibodies, but the intervals will most likely be wider than in common immunoadsorption. This will have to be determined in clinical trials.

6. List of Abbreviations

AP	alkaline phosphatase
Cy2	cyanine 2
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphophates
Dsg1	desmoglein 1
Dsg3	desmoglein 3
E. coli	*Escherichia coli*
EBNA1	Epstein-Barr virus nuclear antigen 1
EBV	Epstein-Barr virus
EC	extracellular domain
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
HRP	horseradish peroxidase
HSP	heat shock protein
IMAC	immobilised metal ion affinity chromatography
kb	kilobasepairs
KGM2	Keratinocyte Growth Medium 2
LB	Luria/Miller
NHS	N-hydroxysuccinimide
p38 MAPK	p38 mitogen-activating protein kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PF	pemphigus foliaceus
PNP	paraneoplastic pemphigus
POD	peroxidase
PV	pemphigus vulgaris
RU	relative units
scFvs	single-chain variable-region fragments
SDS	sodium dodecylsulfate
TM	transmembrane domain
TRITC	tetramethylrhodamine isothiocyanate
U	units

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