

available at www.sciencedirect.com

Clinical Immunology

www.elsevier.com/locate/yclim

**Clinical
Immunology
Society**


Clinical activity of pemphigus vulgaris relates to IgE autoantibodies against desmoglein 3

Angela Nagel ^{a,*}, Angelika Lang ^b, Dorothee Engel ^b, Eva Podstawa ^a,
Nicolas Hunzelmann ^c, Ornella de Pita ^d, Luca Borradori ^e,
Wolfgang Uter ^{f,1}, Michael Hertl ^{a,b,1}

^a Department of Dermatology and Allergology, Philipps University Marburg, Deutschhausstraße 9, D35037 Marburg, Germany

^b Department of Dermatology, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

^c Department of Dermatology and Venerology, University of Cologne, Cologne, Germany

^d Department of Immunodermatology, Istituto Dermatico dell' Immacolata, Rome, Italy

^e Department of Dermatology, University Hospital, Berne, Switzerland

^f Institute of Medical Statistics, Biometry and Epidemiology, Friedrich-Alexander-University Erlangen-Nürnberg, Erlangen, Germany

Received 28 September 2009; accepted with revision 16 November 2009

KEYWORDS

Pemphigus;
Desmoglein 3;
IgG4;
IgE;
Autoantibody;
T helper 2 cell

Abstract Pemphigus vulgaris (PV) is a severe autoimmune bullous skin disease and is primarily associated with IgG against desmoglein 3 (dsg3), a desmosomal adhesion protein. In light of the recent association of autoreactive T helper (Th) 2 cells with active PV, the present study sought to relate the occurrence of Th2-regulated dsg3-specific autoantibody subtypes, i.e. IgE and IgG4, in 93 well-characterized PV patients. Patients with acute onset PV ($n=37$) showed the highest concentrations of serum IgE and IgG4 autoantibodies, which were significantly lower in PV patients in remission ($n=14$). Furthermore, there was a strong correlation between dsg3-reactive IgE and IgG4 in acute onset, but not in chronic active ($n=42$) or remittent patients. Additionally, intercellular IgE deposits were detected in the epidermis of acute onset PV. Thus, dsg3-specific IgE and IgG4 autoantibodies are related to acute onset disease which provides additional support to the concept that PV is a Th2-driven autoimmune disorder.

© 2009 Elsevier Inc. All rights reserved.

Introduction

Pemphigus vulgaris (PV) is a severe, potentially life-threatening autoimmune disease characterized by painful non-healing erosions and flaccid blisters of the skin and/or mucous membranes [1]. Clinical disease is the consequence of the disruption of intercellular adhesion between keratinocytes, known as acantholysis [2], caused by IgG autoantibodies (autoab) against desmosomal components [3].

Abbreviations: autoab, autoantibody; BP, bullous pemphigoid; DIF, direct immunofluorescence; dsg, desmoglein; Ig, immunoglobulin; IIF, indirect immunofluorescence; OD, optical density; PV, pemphigus vulgaris.

* Corresponding author. Fax: +49 6421 2865729.

E-mail address: nagela@med.uni-marburg.de (A. Nagel).

¹ Both authors contributed equally.

1521-6616/\$ - see front matter © 2009 Elsevier Inc. All rights reserved.

doi:[10.1016/j.clim.2009.11.006](https://doi.org/10.1016/j.clim.2009.11.006)

Please cite this article as: A. Nagel, et al., Clinical activity of pemphigus vulgaris relates to IgE autoantibodies against desmoglein 3, Clin. Immunol. (2009), doi:[10.1016/j.clim.2009.11.006](https://doi.org/10.1016/j.clim.2009.11.006)

Table 1 Demographic and clinical characteristics of patients with pemphigus vulgaris (PV).

	PV			
	All	Acute onset	Chronic active	Remittent
Number of PV patients	93	37	42	14
Age [years; median (range)]	52 (1–82)	52 (21–82)	53 (1–65)	47 (25–67)
% Males	46	52	47	31
% Females	54	48	53	69
Disease duration [months; median (range)]	13 (1–192)	1 (1–3)	53 (4–192)	54 (13–144)
<i>Original disease manifestation</i>				
% Skin only	22	27	21	13
% Mucosa only	24	23	18	50
% Skin and mucosa	54	50	61	37
<i>IIF</i>				
% Positive	80.5	85	82	64
% Negative	19.5	15	18	36
Titer [range]	0–1:3200	0–1:3200	0–1:3200	0–1:200
<i>Drug therapy at the time of blood withdrawal [number of patients]</i>				
Prednisone	52	14	30	8
Mycophenolate mofetil	7	1	5	1
Azathioprine	25	5	14	6
Dapsone	2	–	–	2
Methotrexate	2	–	2	–
Cyclophosphamide	3	1	2	–
Cyclosporine	5	1	2	2
IVIg	1	–	1	–
Dexamethasone	1	–	1	–

IVIg – intravenous immunoglobulin, IIF – indirect immunofluorescence.

The precise mechanisms leading to this autoab-mediated epidermal loss of cell–cell adhesion are currently not completely understood. However, autoab-mediated disruption of cell adhesion by direct inhibition of dsg3 transinteraction (steric hindrance) as well as cellular signaling events resulting in reduction of dsg-binding and reorganization of the cytoskeleton of keratinocytes are hypothetically discussed [4–6].

Although autoab production in PV is polyclonal and probably directed against several autoantigens such as desmogleins [7,8] and additional desmosomal and non-desmosomal autoantigens [9], desmoglein 3 (dsg3), a transmembranous adhesion glycoprotein of desmosomes, is considered as the major pathogenic autoantigen of PV

[2,10,11]. The pathogenic potential of dsg3-specific autoab is supported by observations, showing that i) disease activity in PV is mostly correlated with dsg3-specific IgG titers in the patients' sera, ii) newborns of mothers with active PV exhibit blisters due to transplacental transmission of maternal dsg3-specific autoab, predominantly of the IgG4 subtype and iii) pemphigus-like lesions are induced in neonatal mice after transfer of purified serum IgG from patients with active PV [2,12,13]. Tissue-bound and serum dsg3-specific autoab mainly belong to the IgG1 and IgG4 subclasses [3]. Several studies provided evidence for a critical role of dsg3-specific IgG4 in the development of PV [3,4,14–16]. In this regard, serum titers of dsg3-reactive IgG4, but not IgG1, were found to correlate with clinical disease activity in PV patients [17,18].

Table 2 Cutoff values for IgG, IgG1, IgG4, IgA and IgE autoantibodies against desmoglein 3 (dsg3) and their diagnostic properties towards pemphigus vulgaris.

	Youden index	Cutoff value ^a	Sensitivity (95% CI) ^b	Specificity (95% CI) ^b
Anti-dsg3 IgG	0.599	0.424	68.4 (58.2–77.4)	91.5 (82.5–96.8)
Anti-dsg3 IgG1	0.399	0.192	93.4 (86.9–97.3)	46.5 (34.6–58.7)
Anti-dsg3 IgG4	0.501	0.299	64.2 (54.3–73.2)	85.9 (75.6–93.0)
Anti-dsg3 IgA	0.082	0.226	18.1 (11.3–26.8)	90.1 (80.7–95.9)
Anti-dsg3 IgE	0.054	0.148	65.4 (54.0–75.7)	40.0 (28.5–52.4)

^a Cutpoints (optical density, OD) and diagnostic properties of autoantibody quantification as determined by maximization of the Youden index.

^b 95% Exact confidence intervals [CIs] given.

In addition, active PV was found to be mainly associated with dsG3-reactive IgG4, while PV patients in remission preferentially exhibit autoab of the IgG1 subtype [14].

It has been previously shown, that cytokines secreted by T helper 2 (Th2) cells, i.e. IL-4 and IL-13, induce B lymphocytes to secrete IgG4 and IgE [19–21]. In line with these findings, several studies suggested, that dsG3-reactive Th2 cells are directly associated with clinically active PV [22,23]. Moreover, T cell infiltrates from PV skin lesions show a prevalent Th2-like pattern [24] and *in vitro* stimulation of CD4⁺ T cells from PV patients with dsG3-derived peptides preferentially leads to secretion of Th2-like cytokines, including IL-4 and IL-6 [19]. Therefore, there is strong evidence to consider PV as a Th2-regulated autoimmune disorder.

Serological analysis of autoab subtypes in bullous pemphigoid (BP), a distinct autoimmune bullous skin disease associated with a Th2-type immune response [2], identified pathogenetically relevant IgG4 and IgE autoab in the majority of the patients' sera [25–28]. In BP, these Th2-regulated autoab subtypes were found to be related to clinical activity and severity [25,27]. In a previous study with selected sera from PV patients, IgE autoab reactive with dsG3 were occasionally detected by immunoblot analysis in clinically active, but not in remittent, PV [29].

It is presently unclear, whether levels of Th2-regulated dsG3-reactive autoab, i.e. IgE and IgG4, are directly correlated with disease activity in PV. To address this issue, serum levels of anti-dsG3 IgE and IgG4 autoab as well as of dsG3-reactive IgG1 and IgA were analyzed and correlated with the clinical phenotype and activity in a cohort of 93 well-characterized PV patients.

Materials and methods

Patients

Serum samples were obtained from a total of 93 PV patients who were subdivided according to clinical disease activity, i.e. acute onset PV ($n=37$), chronic active PV ($n=42$) and remittent PV ($n=14$). "Acute onset" was defined as the *de novo* development of blisters/erosions on previously unaffected mucosal±cutaneous surfaces with a disease duration of ≤ 3 months. "Chronic active" disease was defined as the expansion/persistence of existing blisters or erosions on mucosal±cutaneous surfaces with a disease duration of ≥ 3 months. PV patients with "remittent" disease had not experienced mucosal±cutaneous erosions ≥ 2 months prior to inclusion in this study. The clinical diagnosis of PV was confirmed by 1) histopathology (suprabasal acantholytic blisters), 2) direct immunofluorescence microscopy (epidermal intercellular IgG and/or C3 deposits in perilesional skin) and/or 3) the detection of serum autoab by indirect immunofluorescence microscopy (intercellular IgG binding to epithelial cells of monkey esophagus).

To investigate the relationship between autoab profiles of PV patients and clinical phenotype, autoab titers in the sera of patients with acute onset, chronic active and remittent disease were compared among each other. All the patients were clinically well-characterized with regard to duration and manifestation of disease (Table 1). Sera were stored at -20°C prior to analysis. The study was conducted according

to Declaration of Helsinki Principles. All samples were obtained following informed consent and approval of the local ethics committee.

Detection of serum autoab by indirect immunofluorescence (IIF)

IIF was performed by a standard technique using monkey esophagus as a substrate according to manufacturer's instructions (monkey esophagus IFA Kit, FK 208.2, The Binding Site, Birmingham, UK). For detection of autoreactive IgG, serum was initially diluted at 1:20 and subsequently titrated in case of positive reaction. Occurrence of serum IgG1, IgG4 and IgE autoab was examined by a modified technique utilizing PE-conjugated mouse anti-human IgG1 (1:100, clone 4E3, Southern Biotech, Birmingham, Alabama, USA), mouse anti-human IgG4 (1:100, clone HP6023, Southern Biotech, Birmingham, Alabama, USA) and FITC-conjugated goat anti-human IgE (1:50, Bethyl, Montgomery, Texas, USA), respectively. For identification of IgG1 and IgG4 autoab serum was diluted at 1:20, whereas IgE autoab were investigated using undiluted serum. The sections were assessed under a fluorescence microscope equipped with standard filters.

Detection of tissue-bound autoab by direct immunofluorescence (DIF)

Experimental proof of tissue-bound IgE was performed by direct immunofluorescent staining of perilesional skin biopsies (cryosections) using an FITC-conjugated goat anti-human IgE antibody (1:50, Bethyl, Montgomery, Texas, USA). Intercellular IgE deposits in the patients' epidermis were visualized by fluorescence microscopy.

Expression and purification of recombinant human dsG3 protein

The recombinant protein PVhis (extracellular domain of dsG3) is linked to a 6× His tag and was produced in an eukaryotic baculovirus expression system and purified over Nickel-NTA agarose as previously described [22,30]. Briefly, 3 days after infection, culture supernatants of 10×10^6 baculovirus-infected insect cells were collected and dialyzed against PBS (pH 7.5) with 0.5 mM calcium chloride (PBS-Ca). DsG3 protein was purified by affinity chromatography over Nickel-NTA agarose (Quiagen, Hilden, Germany) according to the manufacturer's instructions, finally dialyzed against PBS-Ca at 4°C for 24 h and aliquots of PVhis were stored at -80°C . Protein concentration was determined by a commercial kit (DC Protein Assay, Bio-Rad, Munich, Germany) according to a modified protocol by Lowry. The recombinant protein was fractionated by 10% SDS-PAGE and visualized by Coomassie staining and western blot with defined patients' sera (dilution 1:100) to verify proper molecular weight and specific immunoreactivity.

ELISA analysis utilizing baculovirus-derived dsG3

Optimal conditions for the ELISA were established by various chessboard titrations following published guidelines [31].

Affinity-purified recombinant human dsG3 protein was immobilized on 96 well polystyrene plates (Maxisorb Immuno-plate, Nunc, Wiesbaden, Germany) by coating each well with 1 µg PVhis in 100 µl PBS-Ca at 4 °C overnight. After washing with PBS-Ca containing 0.05% Tween-20 (Merck, Nürnberg, Germany) plates were blocked for 2 h with PBS-Ca/T containing 5% skimmed milk powder at room temperature and incubated with patients' sera diluted at 1:50 in blocking-buffer overnight at 4 °C. Each sample was run in duplicate. After consecutive washes as mentioned above, wells were incubated with 6000-fold-diluted alkaline phosphatase (AP)-labeled rabbit anti-human IgG (Dako, Glostrup, Denmark), monoclonal biotinylated mouse anti-human IgG1 (1:800, clone HP6069, Zymed, San Francisco, California, USA), monoclonal biotinylated mouse anti-human IgG4 (1:500, clone HP6025, Zymed, San Francisco, California, USA), biotinylated mouse anti-human IgE (1:500, Zymed, San Francisco, California, USA) or peroxidase-conjugated sheep anti-human IgA (1:5000, The Binding Site, Birmingham, UK) for 1–2 h at room temperature. Incubation with biotinylated antibodies for 1 h was followed by addition of streptavidin-AP (ZyMAX, Zymed, San Francisco, California, USA) diluted at 1:2000 in PBS for 1 h at room temperature. Color development was achieved using 100 µl *p*-nitrophenylphosphate (pNPP, Kirkegaard and Perry, Gaithersburg, Maryland, USA) or the peroxidase substrate 2,2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS, Sigma, Deisenhofen, Germany). Optical density (OD) was measured at 405 nm by ELISA reader (Wallac 1420 microplate reader, Wallac, Freiburg, Germany) when a defined patient serum (positive reference control for IgG) reached an OD of 1.0.

Statistical analysis

In view of the non-normal distribution of OD values, continuous variables are shown as median with quartiles and range, illustrated as box-whisker-plots and non-parametric tests were used. The box contains 50% of the data (1st and 3rd quartile), in which the center line represents the median. The whiskers restrict minimum and maximum of the data set. Outliers (distance from 1st or 3rd quartile >1.5× length of the box) are illustrated as points and extreme values (distance from 1st and 3rd quartile >3× length of the box) as asterisks, respectively. Titers of IIF were log-transformed for further analyses.

For comparison of the three different patient groups (acute onset, chronic active, remittent) the Kruskal–Wallis-test was performed at first. In the case of $p < 0.05$, differences between two groups (acute onset/chronic active, acute onset/remittent and chronic active/remittent) were compared using the two-sided Mann–Whitney-*U*-test in terms of a closing test procedure. Correlation was quantified with the Spearman rank correlation coefficient.

Level of significance α was defined as less than 0.05 ($p < 0.05$). The reported *p*-values were not adjusted for multiple testing. Statistical analysis was performed using SPSS.

Receiver operating characteristic (ROC) curves were compiled in consideration of PV patients and healthy controls by additionally using SAS (version 9.1, SAS Institute, Cary, NC) to evaluate the diagnostic properties of different dsG3 antibodies with respect to correctly classifying cases of PV.

As there are no “natural” cutpoints for dsG3 antibody levels, and as there are no previous studies which could have contributed evidence concerning an appropriate diagnostic threshold, an “optimum” cutpoint was determined data-adaptively by choosing the OD values with the largest Youden index ($YI = \text{sensitivity} + \text{specificity} - 1$). The corresponding sensitivity and specificity were estimated including exact 95% confidence intervals calculated with the Blyth–Still–Casella method. Seroreactivity determined by ELISA was considered positive, if the OD value was above the respective cutpoint (Table 2).

Results

Age and sex distribution and basic data of studied PV patients

Among the 93 PV patients, there were 46% males and 54% females (male to female ratio 1:1.15). Table 1 summarizes the pattern of clinical involvement, disease duration, range of IgG autoab titers determined by IIF as well as the administered therapy including corticosteroids, immunosuppressive and immunomodulatory drugs, respectively, of all the PV patients subdivided according to the disease stage at the time of blood withdrawal.

Autoab in PV patients in association with sex and relationship to clinical stage of disease

Investigation of the 93 PV patients did not show any gender-specific predisposition for disease development. As illustrated in Table 3, neither autoab titers nor age and disease duration differed significantly between male and female patients. In addition, subdividing groups of male and female patients according to their clinical disease stage (acute onset, chronic active, remittent) did not reveal statistical significant differences between both sexes (data not shown).

With regard to the concentrations of dsG3-specific total IgG including IgG1 and IgG4, IgA and IgE autoab, statistically significant differences between the patients groups (acute onset, chronic active, remittent) were observed. Fig. 1 illustrates the markedly lower levels of total IgG (Fig. 1a),

Table 3 Gender-specific characteristics of pemphigus vulgaris (PV) patients.

	PV patients		<i>p</i> ^a
	Males	Females	
Age [years, median]	53	51	0.476
Anti-dsG3 IgG [OD, median]	0.619	0.572	0.836
Anti-dsG3 IgG1 [OD, median]	0.336	0.325	0.953
Anti-dsG3 IgG4 [OD, median]	0.346	0.333	0.447
Anti-dsG3 IgA [OD, median]	0.156	0.157	0.563
Anti-dsG3 IgE [OD, median]	0.199	0.180	0.450
IIF IgG [log 10 titer, median]	2.00	1.70	0.253
Disease duration [months, median]	13	15	0.994

OD – optical density; dsG – desmoglein; IIF – indirect immunofluorescence.

^a Statistical comparison of the groups (male and female PV patients) was evaluated using two-sided Mann–Whitney-*U*-test.

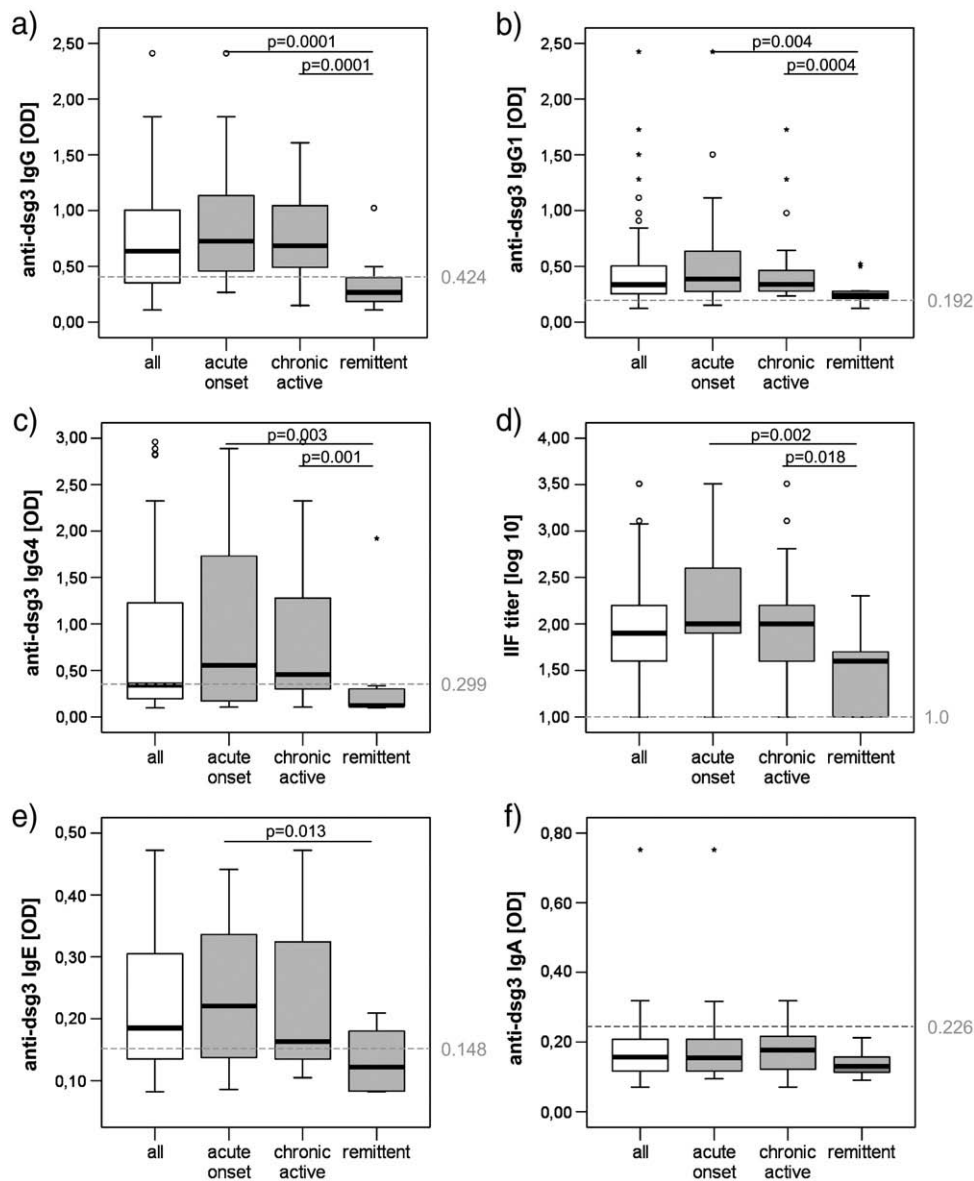


Figure 1 Relationship between autoantibody (autoab) subtypes against desmoglein 3 (dsg3) and the clinical stage of pemphigus vulgaris (PV). Fig. 1 demonstrates pronounced differences in the concentrations of dsg3-reactive autoab depending on the clinical stage of PV. Dsg3-specific serum total IgG (a), IgG1 (b) and IgG4 (c) were significantly lower in remittent PV compared to acute onset and chronic active PV, respectively. Analysis of IgG autoab by indirect immunofluorescence (data are presented as log values) confirmed these results (d). Dsg3-specific IgE was most prominent in acute onset PV and showed a significant lower level in remittent PV (e). In contrast, dsg3-reactive serum IgA was not detectable in the majority of the patients independent from the disease stage (f). Using Mann–Whitney–U-test, statistically significant pair-wise differences between patients groups are shown above the respective boxplots.

IgG1 (Fig. 1b) and IgG4 (Fig. 1c) concentrations in PV patients in clinical remission compared to patients with acute onset or chronic active PV, respectively. Overall, as shown in Fig. 1d, levels of IgG autoab, determined by IIF using monkey esophagus as substrate, were much lower in remittent patients (titer range=0–1:200, Table 1) compared to acute onset and chronic active PV, respectively (titer range=0–1:3200, Table 1).

Noteworthy, the highest concentrations of dsg3-reactive serum IgE were found in patients with acute onset PV and were significantly lower in clinical remission (Fig. 1e). In contrast, IgA autoab were only detected at low concentra-

tions in a minority of patients (15/93) and did not show any correlation with the clinical course of PV (Fig. 1f). Importantly, the observed significant decline in the serum concentrations of dsg3-reactive total IgG (Figs. 1a, d), IgG1 (Fig. 1b), IgG4 (Fig. 1c) and IgE (Fig. 1e) between the groups of acute onset/chronic active and remittent PV was not attributed to varying disease duration among the groups. As shown in Fig. 2 for dsg3-specific IgG, distribution of the 93 PV patients with regard to their individual disease duration (independent of clinical stage) did not result in a statistically significant difference of the aforementioned autoab titers (Kruskal–Wallis-test, Fig. 2a: $p=0.312$ (IgG); not shown:

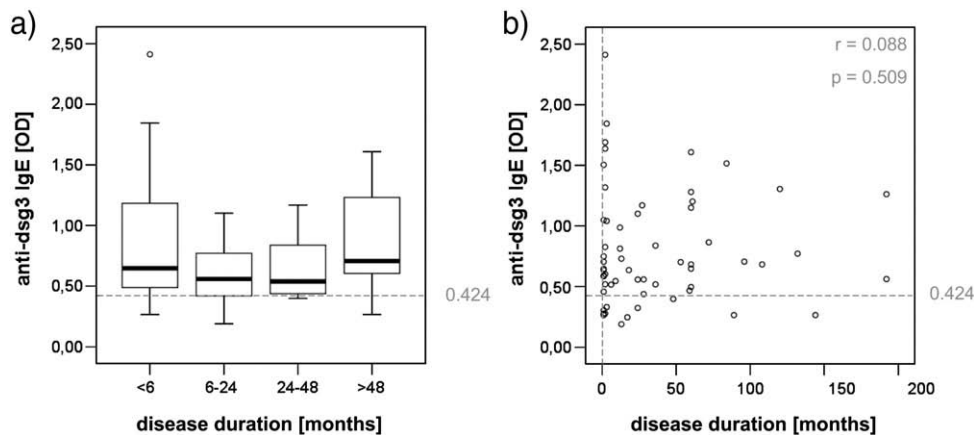


Figure 2 Relationship between desmoglein 3 (dsg3)-reactive IgG and disease duration in patients with pemphigus vulgaris (PV). Illustrated are the levels of dsg3-specific serum IgG in the 93 PV patients, who were subdivided regarding disease duration (a). Correlation of dsg3-reactive IgG and disease duration of PV was also evaluated (b). Both analyses did not show statistically significant differences.

$p=0.837$ (IgG1), $p=0.541$ (IgG4), $p=0.938$ (IgE)). Moreover, a correlation between the amount of serum autoab and disease duration (Fig. 2b: $p=0.509$ (IgG), not shown: $p=0.832$ (IgG1), $p=0.234$ (IgG4), $p=0.695$ (IgE)) was not detected in the investigated patients. Furthermore, lower amounts of IgG, IgG1, IgG4 and IgE autoab in remittent patients were apparently not a result of treatment. Distribution of patients into subgroups based on whether they were on treatment or not did not reveal statistically significant differences between patients groups, neither on examination of all patients nor of patients classified according to their stage of disease (data not shown).

Twenty-four percent of the PV patients had disease limited to mucosal lesions (mostly oral cavity), 22% of the patients showed exclusive cutaneous involvement, while the other 54% of the patients exhibited a more generalized disease with cutaneous and mucosal lesions (Table 1). However, division of the PV patients regarding their initial disease manifestation did not reveal any obvious disparity of

distinct dsg3-specific autoab subtypes between the three groups (data not shown). Consistently, statistically significant differences in serum autoab levels after classification of the patients groups (acute onset, chronic active, remittent) regarding cutaneous, mucosal or mucocutaneous involvement, respectively, were not detectable (data not shown).

Relationship of dsg3-specific IgG1, IgG4 and IgE autoab in PV patients to clinical stage of disease

IgG1 and IgG4 have been shown to be the major subtypes of dsg3-specific IgG in PV [3]. In this regard, IgG4 autoab were found predominantly in active PV, whereas IgG1 was mainly associated with remittent disease [2,14]. Individual proportions of serum concentrations of the dsg3-specific, Th1-linked IgG1 subtype and Th2-related IgG4 and IgE autoab subjected to different disease stages were assessed by calculating distinct ratios (Fig. 3). The ratio of autoreactive IgG1/IgG4 was significantly elevated in remittent PV

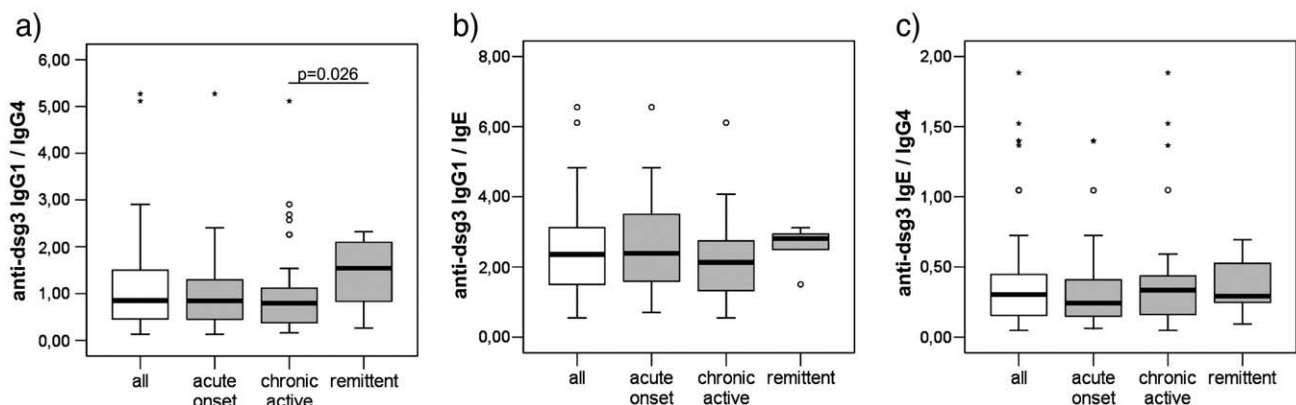


Figure 3 Relationship of desmoglein 3 (dsg3) reactive IgG1, IgG4 and IgE autoantibodies in different clinical stages of pemphigus vulgaris (PV). Shown are the ratios of dsg3-specific IgG1/IgG4 (a), IgG1/IgE (b) and IgE/IgG4 (c) at different clinical stages of PV. The ratios of Dsg3-reactive IgG1/IgG4 as well as IgG1/IgE were apparently higher in remittent PV (a, b), while the IgE/IgG4 ratio did not show differences in any of the disease stages (c). Thus, the increment of the IgG1/IgG4 and IgG1/IgE ratio was inversely correlated with disease activity. Using the Mann–Whitney–U-test, statistically significant pair-wise differences between patients groups are shown above the respective boxplots.

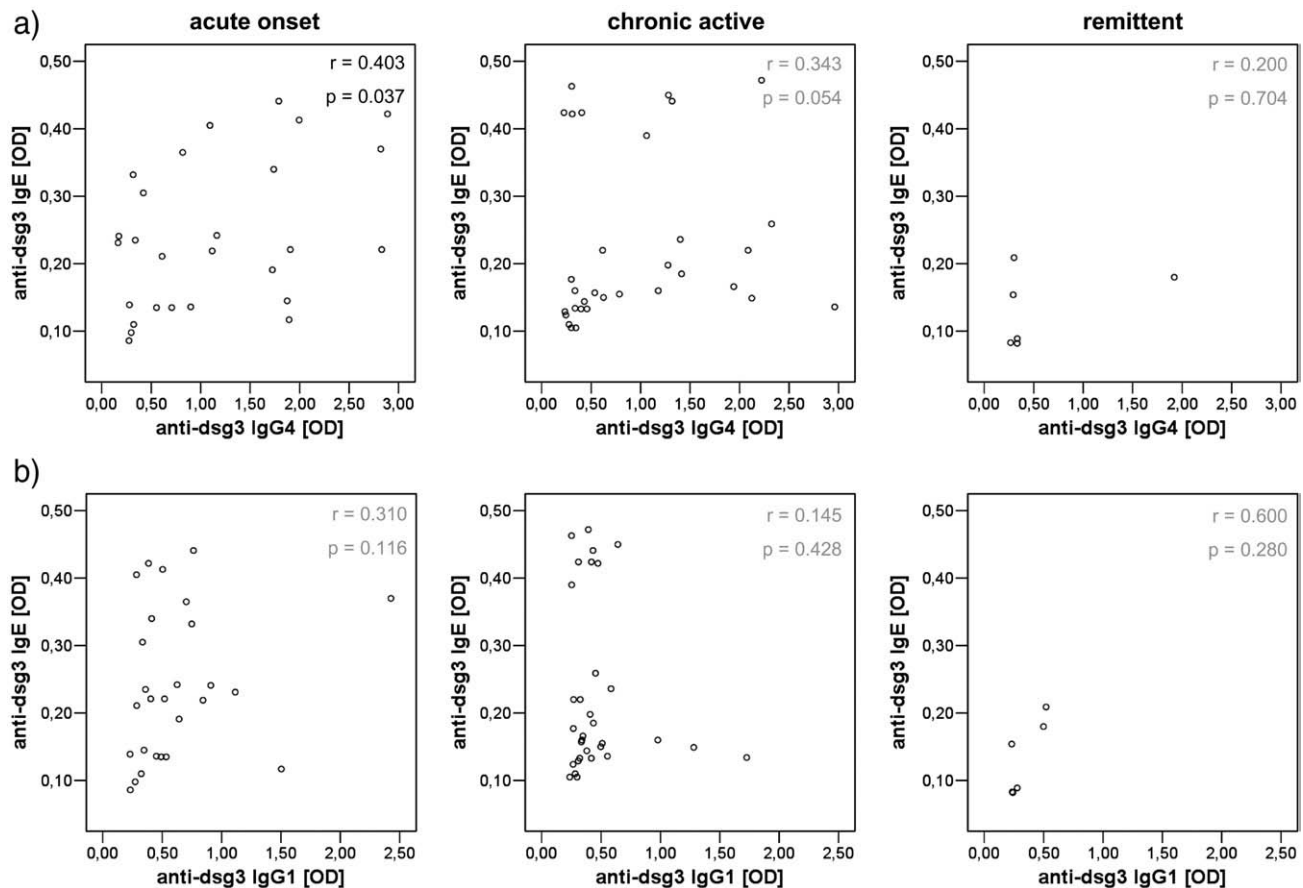


Figure 4 Correlation between desmoglein 3 (dsg3)-specific IgE and IgG4 in different clinical stages of pemphigus vulgaris (PV). Shown is the correlation between dsg3-reactive IgE and IgG4 (a) and IgE and IgG1 autoantibodies (b), respectively. Analysis of the PV patients revealed a statistically significant correlation between dsg3-reactive IgE and IgG4 only in the group of patients with acute onset disease (a, left row), but not in chronic active or remittent PV, respectively (a, center, right). Dsg3-specific IgE and IgG1 autoantibodies were unrelated in any of the disease stages (b).

(Fig. 3a). Since both dsg3-reactive IgG1 (Fig. 1b) and IgG4 (Fig. 1c) autoab were significantly diminished in remittent PV, it is obvious that the decrease of IgG4 exceeded the decline of IgG1 in these patients resulting in the observed elevated IgG1/IgG4 ratio. Overall, there were no statistically significant differences in the ratios of dsg3-specific IgG subtypes between acute onset versus chronic active PV; this finding is presumably owing to the active stage of disease in both groups. Similarly, the ratio of IgG1/IgE autoab showed a tendency to increase upon clinical remission of the studied PV patients (Fig. 3b). As expected, the proportion of Th2-related IgE/IgG4 autoab was not different between PV patients who were subdivided regarding their clinical disease stage, reflecting consistent properties of these autoab independent of disease stage (Fig. 3c).

Direct correlation of Th2-related IgG4 and IgE autoab with acute onset PV

Fig. 4 illustrates the correlation of serum IgE and IgG4 (Fig. 4a) or IgG1 (Fig. 4b) autoab concentrations in the three clinical stages of the studied PV patients. Statistical analysis disclosed a significant correlation between the concentrations of dsg3-specific IgG4 and IgE autoab only in acute onset

(Fig. 4a, left row), but not in chronic active or remittent PV (Fig. 4a, center/right row), reflecting an exclusive association of Th2-related IgG4 and IgE autoab with acute onset disease. In contrast, dsg3-specific IgG1 autoab were not significantly related to dsg3-reactive IgE in any of the disease stages (Fig. 4b).

Detection of circulating and tissue-bound IgE autoab by immunofluorescence

To confirm the ELISA results, occurrence of circulating and tissue-bound IgE autoab was assessed by IIF and DIF, respectively. Shown are the findings of a PV patient with primarily acute onset disease (Figs. 5 and 6a), fading after initiation of corticosteroid treatment to a chronic active disease stage (Fig. 6b) and later on to remission (Fig. 6c). Fig. 5 illustrates clinical and histological findings of an untreated, newly diagnosed (acute onset) PV patient showing flaccid blisters and exudative erosions of the skin (Fig. 5a) and mucous membranes. Histopathology of perilesional skin revealed infiltration of lymphocytes including eosinophils (eosinophilic spongiosis) in the epidermis and superficial dermis (Fig. 5b). Additionally, acantholysis and binding of IgE autoab on the surface of epidermal keratinocytes could be

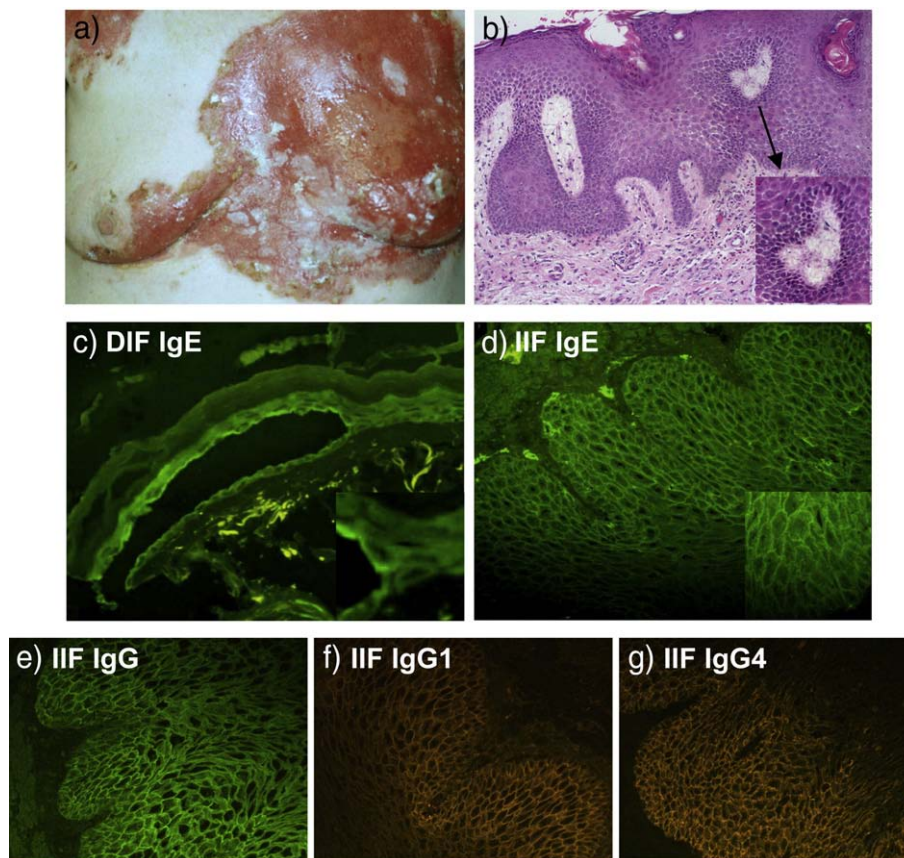


Figure 5 Tissue-bound IgE autoantibodies in acute onset pemphigus vulgaris (PV). Illustrated is a patient with acute onset PV clinically characterized by blisters and extensive erosions of the skin (a) and mucous membranes (not shown). Histological staining of perilesional skin biopsy disclosed infiltration of lymphocytes and eosinophils in the epidermis and superficial dermis (b). Acantholysis and IgE binding on epidermal keratinocytes could be shown by direct immunofluorescent staining of perilesional skin (c). Moreover, investigation of serum autoantibodies by indirect immunofluorescence using monkey esophagus resulted in detection of IgE (d, undiluted serum), IgG (e, serum 1:20), IgG1 (f, serum 1:20) and IgG4 (g, serum 1:20) autoantibodies.

shown by direct immunofluorescent staining of perilesional skin (Fig. 5c). Detection of serum IgE (Figs. 5d and 6a, right), IgG (Figs. 5e and 6a, left), IgG1 (Fig. 5f) and IgG4 (Fig. 5g) autoab by IIF using monkey esophagus disclosed the typical intercellular staining pattern of PV. Treatment with prednisolone and azathioprine over 5 months led to marginal clinical improvement accompanied by a chronic active disease stage, in which IgG (Fig. 6b, left), but not IgE (Fig. 6b, right) autoab were detectable. Similarly, IgG (Fig. 6c, left), but not IgE (Fig. 6c, right) autoab could be found at time of clinical remission thirty months after diagnosing PV.

Irrespective of the restricted sensitivity and specificity of the ELISA system for detection of dsG3-specific IgE (Table 2), present analyses of a representative PV patient, who was observed over a long period of time and therefore characterized in all three clinical stages, confirm the ELISA findings of IgE autoab mainly in acute onset (initial phase) and not in chronic active or remittent PV.

Discussion

In the present study, we show for the first time that, in addition to IgG4, IgE autoab reactive with the major

autoantigen of PV, dsG3, are present in a significant number of patients with acute onset and active PV. Moreover, tissue-bound and serum IgE autoab were detected in acute onset PV by direct and indirect immunofluorescence microscopy. Furthermore, both dsG3-reactive IgG4 and IgE levels were exclusively correlated with acute onset, but not remittent, PV. This finding provides additional support for the concept that autoreactive Th2 cells and Th2-related cytokines are critically involved in the initiation and perpetuation of the autoimmune pathogenesis of PV [19].

Several studies identified dsG3-specific Th2 cells in patients with active PV but not in healthy carriers of PV-associated HLA class II alleles [19,22–24]. A dichotomy of pathogenic autoreactive Th2 cells in diseased patients and Th1 cells in healthy individuals was also noted in pemphigus foliaceus, a clinical variant of pemphigus caused by IgG autoab against dsG1 [32]. Moreover, the presence of Th2-regulated IgG4 autoab by autoreactive B cells and plasma cells is a hallmark of acute/active PV and presumably a key event in the development of clinically apparent PV [3,14]. In contrast, PV patients in remission exhibit strongly elevated IgG1 autoab [14]. Class switch to IgG1 is usually driven by Th1 cells, which have been found to be increased in PV patients with less acute disease and in healthy individuals [22]. IL-4

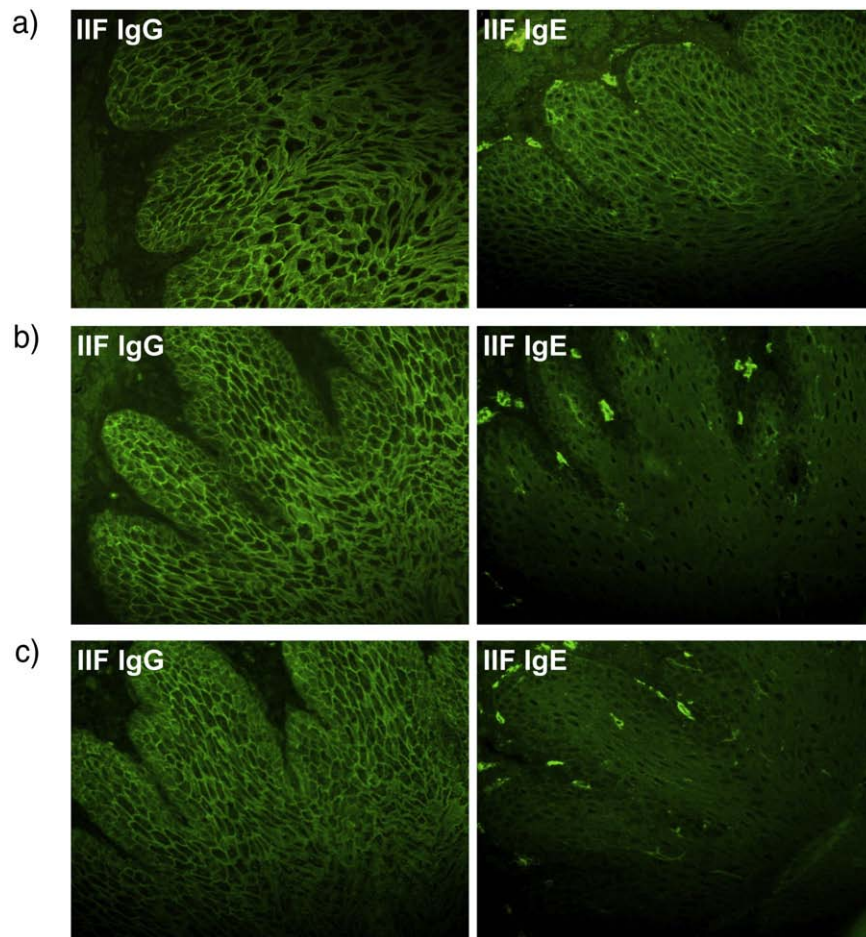


Figure 6 Clinical improvement is accompanied by loss of autoreactive IgE antibodies. Indirect immunofluorescence using monkey esophagus disclosed both IgG (a, left, serum 1:20) and IgE (a, right, undiluted serum) autoantibodies in serum of an untreated PV patient with acute onset disease. Analyses of serum autoantibody titers after immunosuppressive treatment of the patient did not reveal IgE autoantibodies in chronic active (b, right, undiluted serum) and remittent (c, right, undiluted serum) disease stage, respectively. In contrast, IgG autoantibodies were detectable in both chronic active (b, left, serum 1:20) and remittent (c, left, serum 1:20) PV.

and IL-13, which are important cytokines released by Th2 cells, are potent inducers not only of IgG4 but also of IgE switching [20,21]. Consistently, the results of this study revealed highest titers of dsG3-reactive IgE autoab in acute onset PV and showed a significant correlation between IgE and IgG4 autoab in these patients. In contrast, IgE and IgG4 autoab were no longer significantly correlated to each other in chronic active or remittent PV. Thus, the detection of the highest serum concentrations of IgE autoab and the close correlation of Th2-regulated dsG3-specific IgE and IgG4 in acute onset PV support the central role of autoreactive Th2 cells [19,33].

Recently, elevated numbers of natural killer cells were detected in patients with active PV, which were found to release the IgE-promoting cytokine IL-5 [34]. Moreover, it has been shown, that CD40 is a central player in the regulation of IgE production [35]. In two independent mouse models of PV, the interaction between autoreactive B and T cells using the CD40-CD40L signaling pathway was shown to be a crucial event for the induction of pathogenic autoab production [36,37]. Thus, the detection of dsG3-specific IgE in active PV, as shown by ELISA, DIF and IIF, may

be linked to the CD40-CD40L-mediated interaction of autoreactive T and B cells. Importantly, the reduction of Th2-regulated IgG4 and IgE autoab in remittent PV is not due to longer disease duration and, consequently, the need for longer treatment periods, since significant differences in autoab titers were not found to be correlated with disease duration (Fig. 2).

With regard to the overall pathogenetic relevance of Th2-regulated autoab in PV, recent studies identified dsG3-specific IgG4 as the main pathogenic IgG subclass in PV [14,15]. A monoclonal dsG3-reactive IgG4 antibody, isolated from a patient with active PV, caused intraepidermal suprabasal blister formation in an *in vitro* organ culture model with normal human skin and led to the development of bullae and superficial erosions in an *in vivo* PV mouse model [15]. In contrast to dsG3-specific IgG4, autoreactive IgG1 failed to bind to human skin presumably due to low avidity supporting the concept of rather non-pathogenic properties of IgG1 in PV [16] and confirming the findings of a predominance of IgG1 autoab in remittent PV. Furthermore, in a previous study, autoreactive IgG1 and IgG4 were found to recognize different epitopes of dsG3 [14].

Recently, Spaeth et al. showed that IgA autoab were detected by immunoblot analysis in most patients with acute onset and chronic active PV, respectively [29]. However, in the present study, systematic investigation by ELISA revealed dsG3-reactive serum IgA only in a minority of the 93 studied PV patients. Moreover, the presence of dsG3-reactive IgA autoab was not correlated with the clinical stage of PV. These results confirm the well-known clinical disparity between PV and IgA pemphigus, a clinically distinct pemphigus variant. Specifically, the intraepidermal neutrophilic dermatosis type of IgA pemphigus has been associated with IgA reactivity against dsG3.

The results of the present study in PV are in line with previous findings in BP, an immunologically related but distinct autoimmune bullous skin disorder [25,27,38]. Autoab production in BP is presumably regulated by autoreactive Th cells that preferentially belong to the Th2 subset [39]. In BP, IgE autoab against BP180, the major autoantigen of BP, were positively correlated with the levels of IgG4 autoab and were directly associated with the clinical activity of BP [27]. This finding is in accordance with the results of the present study and may have implications for the therapeutic targeting of the IgE-mediated pathology in PV. Fairley et al. demonstrated for the first time *in vivo* that IgE autoab from BP sera induced dermoepidermal separation of human skin grafted onto nude mice [40]. The molecular mode of action of IgE autoab in BP, including the involvement of Fcε-positive cutaneous mast cells, remains to be further analyzed [28]. Along this line, Fairley et al. reported the successful treatment of a patient with refractory BP with omalizumab, a monoclonal antibody against human IgE [41]. As dsG3-specific IgE is preferentially found in acute onset PV (present findings, [29]), administration of omalizumab may provide a therapeutic option in early PV in cases in whom immunosuppressive therapy is contraindicated. Further studies are needed to substantiate the role of IgE autoab in the pathogenesis of PV.

In summary, the close association of Th2-regulated IgG4 and IgE autoab with clinically active PV observed in the present study provides additional insights for potential novel therapeutic strategies in PV, i.e. targeting of dsG3-specific autoreactive Th2 cells and/or IgE.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

Financial support for the study was provided by Deutsche Forschungsgemeinschaft (He 1602/8-1 and 1602/8-2 to M.H. and N.H.) and a grant from the Universitätsklinikum Giessen und Marburg GmbH, Marburg, Germany (UKGM, to M.H.). Furthermore, this project was supported by Coordination Theme 1 (Health) of the European Community's FP7, Grant agreement number HEALTH-F2-2008-200515 (to M.H.).

References

- [1] J.C. Bystryń, J.L. Rudolph, Pemphigus, *Lancet* 366 (2005) 61–73.
- [2] M. Hertl, Humoral and cellular autoimmunity in autoimmune bullous skin disorders, *Int. Arch. Allergy Immunol.* 122 (2000) 91–100.
- [3] C. Sitaru, S. Mihai, D. Zillikens, The relevance of the IgG subclass of autoantibodies for blister induction in autoimmune bullous skin diseases, *Arch. Dermatol. Res.* 299 (2007) 1–8.
- [4] D. Ioannides, E. Lazaridou, D. Rigopoulos, Pemphigus, *J. Eur. Acad. Dermatol. Venereol.* 22 (2008) 1478–1496.
- [5] J. Waschke, The desmosome and pemphigus, *Histochem. Cell. Biol.* 130 (2008) 21–54.
- [6] W.M. Heupel, D. Zillikens, D. Drenckhahn, J. Waschke, Pemphigus vulgaris IgG directly inhibit desmoglein 3-mediated transinteraction, *J. Immunol.* 181 (2008) 1825–1834.
- [7] M. Amagai, Autoimmunity against desmosomal cadherins in pemphigus, *J. Dermatol. Sci.* 20 (1999) 92–102.
- [8] R. Moll, I. Moll, Epidermal adhesion molecules and basement membrane components as target structures of autoimmunity, *Virchows Arch.* 432 (1998) 487–504.
- [9] S.A. Grando, Autoimmunity to keratinocyte acetylcholine receptors in pemphigus, *Dermatology* 201 (2000) 290–295.
- [10] M. Amagai, V. Klaus-Kovtun, J.R. Stanley, Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion, *Cell* 67 (1991) 869–877.
- [11] M. Amagai, S. Karpatis, R. Prussick, V. Klaus-Kovtun, J.R. Stanley, Autoantibodies against the amino-terminal cadherin-like binding domain of pemphigus vulgaris antigen are pathogenic, *J. Clin. Invest.* 90 (1992) 919–926.
- [12] T. Parlow, J. Welzel, M. Amagai, D. Zillikens, T. Wygold, Neonatal pemphigus vulgaris: IgG4 autoantibodies to desmoglein 3 induce skin blisters in newborns, *J. Am. Acad. Dermatol.* 48 (2003) 623–625.
- [13] M. Amagai, A.R. Ahmed, Y. Kitajima, J.C. Bystryń, Y. Milner, R. Gnidecki, M. Hertl, C. Pincelli, H. Kurzen, M. Fridkis-Hareli, Y. Aoyama, M. Frusic-Zlotkin, E. Muller, M. David, D. Mimouni, D. Vind-Kezunovic, B. Michel, M. Mahoney, S. Grando, Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just “witnesses of disease”? *Exp. Dermatol.* 15 (2006) 815–831.
- [14] K. Bhol, K. Natarajan, N. Nagarwalla, A. Mohimen, V. Aoki, A. R. Ahmed, Correlation of peptide specificity and IgG subclass with pathogenic and nonpathogenic autoantibodies in pemphigus vulgaris: a model for autoimmunity, *Proc. Natl. Acad. Sci. U. S. A.* 92 (1995) 5239–5243.
- [15] S.W. Yeh, L.A. Cavacini, K.C. Bhol, M.S. Lin, M. Kumar, M. Duval, M.R. Posner, A.R. Ahmed, Pathogenic human monoclonal antibody against desmoglein 3, *Clin. Immunol.* 120 (2006) 68–75.
- [16] M.K. Hacker, M. Janson, J.A. Fairley, M.S. Lin, Isotypes and antigenic profiles of pemphigus foliaceus and pemphigus vulgaris autoantibodies, *Clin. Immunol.* 105 (2002) 64–74.
- [17] A.R. Ahmed, Z. Spigelman, L.A. Cavacini, M.R. Posner, Treatment of pemphigus vulgaris with rituximab and intravenous immune globulin, *N. Engl. J. Med.* 355 (2006) 1772–1779.
- [18] R. Eming, A. Nagel, S. Wolff-Franke, E. Podstawa, D. Debus, M. Hertl, Rituximab exerts a dual effect in pemphigus vulgaris, *J. Invest. Dermatol.* 128 (2008) 2850–2858.
- [19] M.S. Lin, S.J. Swartz, A. Lopez, X. Ding, M.A. Fernandez-Vina, P. Stastny, J.A. Fairley, L.A. Diaz, Development and characterization of desmoglein-3 specific T cells from patients with pemphigus vulgaris, *J. Clin. Invest.* 99 (1997) 31–40.
- [20] R.S. Geha, H.H. Jabara, S.R. Brodeur, The regulation of immunoglobulin E class-switch recombination, *Nat. Rev. Immunol.* 3 (2003) 721–732.
- [21] R.C. Aalberse, S.O. Stapel, J. Schuurman, T. Rispens, Immunoglobulin G4: an odd antibody, *Clin. Exp. Allergy* 39 (2009) 469–477.
- [22] C. Veldman, A. Stauber, R. Wassmuth, W. Uter, G. Schuler, M. Hertl, Dichotomy of autoreactive Th1 and Th2 cell responses to

- desmoglein 3 in patients with pemphigus vulgaris (PV) and healthy carriers of PV-associated HLA class II alleles, *J. Immunol.* 170 (2003) 635–642.
- [23] C. Rizzo, M. Fotino, Y. Zhang, S. Chow, A. Spizuoco, A.A. Sinha, Direct characterization of human T cells in pemphigus vulgaris reveals elevated autoantigen-specific Th2 activity in association with active disease, *Clin. Exp. Dermatol.* 30 (2005) 535–540.
- [24] M. Caproni, B. Giomi, C. Cardinali, E. Salvatore, E. Pestelli, A. D'Agata, B. Bianchi, P. Toto, C. Feliciani, P. Fabbri, Further support for a role for Th2-like cytokines in blister formation of pemphigus, *Clin. Immunol.* 98 (2001) 264–271.
- [25] R. Dopp, E. Schmidt, I. Chimanovitch, M. Leverkus, E.B. Brocker, D. Zillikens, IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in Bullous pemphigoid: serum levels of these immunoglobulins reflect disease activity, *J. Am. Acad. Dermatol.* 42 (2000) 577–583.
- [26] S.K. Dresow, C. Sitaru, A. Recke, G.J. Oostingh, D. Zillikens, B. F. Gibbs, IgE autoantibodies against the intracellular domain of BP180, *Br. J. Dermatol.* 160 (2009) 429–432.
- [27] Y. Iwata, K. Komura, M. Kodera, T. Usuda, Y. Yokoyama, T. Hara, E. Muroi, F. Ogawa, M. Takenaka, S. Sato, Correlation of IgE autoantibody to BP180 with a severe form of bullous pemphigoid, *Arch. Dermatol.* 144 (2008) 41–48.
- [28] O.G. Dimson, G.J. Giudice, C.L. Fu, F. Van den Bergh, S.J. Warren, M.M. Janson, J.A. Fairley, Identification of a potential effector function for IgE autoantibodies in the organ-specific autoimmune disease bullous pemphigoid, *J. Invest. Dermatol.* 120 (2003) 784–788.
- [29] S. Spaeth, R. Riechers, L. Borradori, D. Zillikens, L. Budinger, M. Hertl, IgG, IgA and IgE autoantibodies against the ectodomain of desmoglein 3 in active pemphigus vulgaris, *Br. J. Dermatol.* 144 (2001) 1183–1188.
- [30] K. Ishii, M. Amagai, R.P. Hall, T. Hashimoto, A. Takayanagi, S. Gamou, N. Shimizu, T. Nishikawa, Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins, *J. Immunol.* 159 (1997) 2010–2017.
- [31] D.M. Kemeny, Titration of antibodies, *J. Immunol. Methods* 150 (1992) 57–76.
- [32] K.L. Gebhard, C.M. Veldman, R. Wassmuth, E. Schultz, G. Schuler, M. Hertl, Ex vivo analysis of desmoglein 1-responsive T-helper (Th) 1 and Th2 cells in patients with pemphigus foliaceus and healthy individuals, *Exp. Dermatol.* 14 (2005) 586–592.
- [33] H. Takahashi, M. Amagai, T. Nishikawa, Y. Fujii, Y. Kawakami, M. Kuwana, Novel system evaluating in vivo pathogenicity of desmoglein 3-reactive T cell clones using murine pemphigus vulgaris, *J. Immunol.* 181 (2008) 1526–1535.
- [34] H. Takahashi, M. Amagai, A. Tanikawa, S. Suzuki, Y. Ikeda, T. Nishikawa, Y. Kawakami, M. Kuwana, T helper type 2-biased natural killer cell phenotype in patients with pemphigus vulgaris, *J. Invest. Dermatol.* 127 (2007) 324–330.
- [35] L.A. Iciek, S.A. Delphin, J. Stavnezer, CD40 cross-linking induces Ig epsilon germline transcripts in B cells via activation of NF-kappaB: synergy with IL-4 induction, *J. Immunol.* 158 (1997) 4769–4779.
- [36] M. Aoki-Ota, M. Kinoshita, T. Ota, K. Tsunoda, T. Iwasaki, S. Tanaka, S. Koyasu, T. Nishikawa, M. Amagai, Tolerance induction by the blockade of CD40/CD154 interaction in pemphigus vulgaris mouse model, *J. Invest. Dermatol.* 126 (2006) 105–113.
- [37] R. Eming, M. Hertl, J. Schmidt, E. Podstawa, C. Feliciani, G. Sonderstrup, T-B interaction is crucial for the induction of desmoglein3-specific IgG autoantibodies in an HLA-class II transgenic mouse model of pemphigus vulgaris, *J. Invest. Dermatol.* 127 (2007) S66.
- [38] E. Delaporte, A. Dubost-Brama, R. Ghohestani, J.F. Nicolas, J.L. Neyrinck, H. Bergoend, A. Janin, M. Capron, IgE autoantibodies directed against the major bullous pemphigoid antigen in patients with a severe form of pemphigoid, *J. Immunol.* 157 (1996) 3642–3647.
- [39] L. Budinger, L. Borradori, C. Yee, R. Eming, S. Ferencik, H. Grosse-Wilde, H.F. Merk, K. Yancey, M. Hertl, Identification and characterization of autoreactive T cell responses to bullous pemphigoid antigen 2 in patients and healthy controls, *J. Clin. Invest.* 102 (1998) 2082–2089.
- [40] J.A. Fairley, C.T. Burnett, C.L. Fu, D.L. Larson, M.G. Fleming, G.J. Giudice, A pathogenic role for IgE in autoimmunity: bullous pemphigoid IgE reproduces the early phase of lesion development in human skin grafted to nu/nu mice, *J. Invest. Dermatol.* 127 (2007) 2605–2611.
- [41] J.A. Fairley, C.L. Baum, D.S. Brandt, K.A. Messingham, Pathogenicity of IgE in autoimmunity: successful treatment of bullous pemphigoid with omalizumab, *J. Allergy Clin. Immunol.* 123 (2009) 704–705.