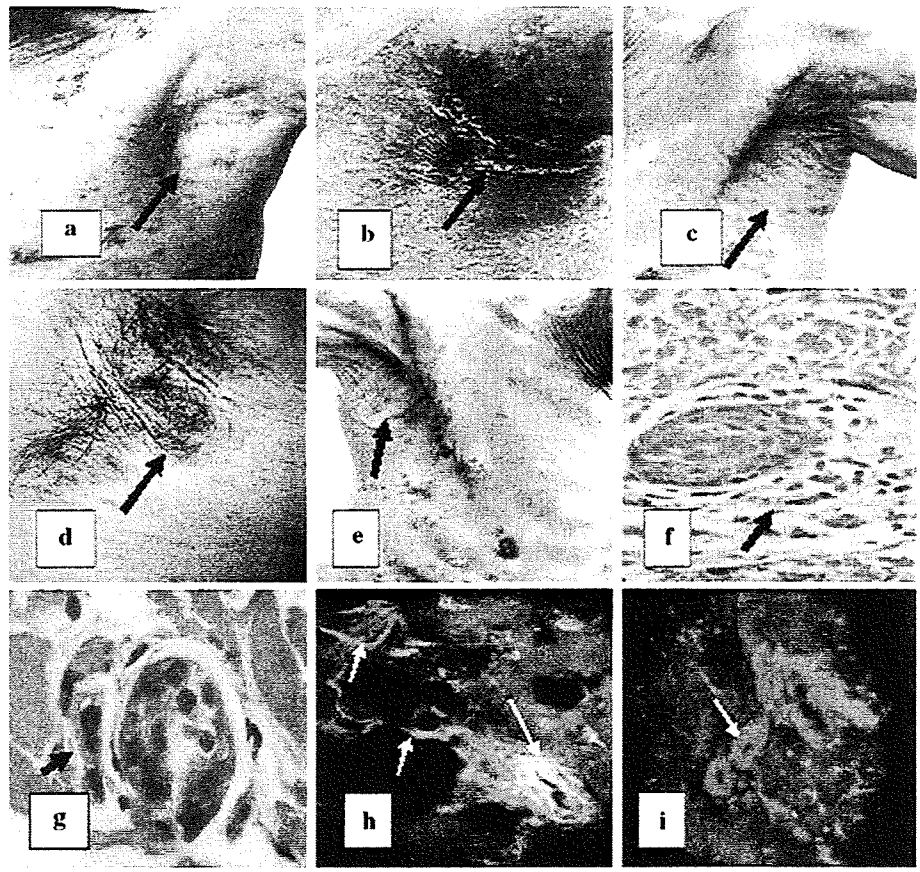


Fig. 1 **a–e** Extended series of broad clinical findings on the axillae of five different El Bagre-EPF patients, including hyperpigmentation, blisters, plaques, and esfacelations on erythematous base. **f** H&E staining demonstrating a squamatized acrosyringium of sweat glands with peri inflammatory infiltrate of mainly lymphohistocytes (×20). **g** A coiled glandular part of the sweat glands with peri inflammatory infiltrate at higher magnification (×40). **h** DIF of El Bagre-EPF axillae showed positive reactivity to the sweat glands when using FITC-conjugated anti-human IgG antibodies. The positive staining around the sweat glands is *yellowish-green* (*yellow arrows*). Note that the nuclei were counterstained with TOPRO-3® (*red dots*). **i** DIF of another El Bagre EPF-patient with positive red staining (*yellow arrows*), as their anti-human IgG was conjugated to rhodamine

CD20 and CD68), or inside the sweat glands (CAE). This confirmed that sweat glands were suffering a specific immunological attack. Figure 2d shows an H&E staining displaying the presence of peri-inflammatory infiltrate around all the sweat glands with a predominance of lymphohistocytes. In Fig. 2c, g, i and j, we were able to observe that, in patients suffering for more than 6 months from El Bagre EPF (chronic cases), the immunoreactivity to the glands decreased due to the reduction or complete destruction of these glands in addition to other deep appendices. This is shown for the concomitant sclerodermoid and sclerodermatous alterations seen in the deep dermis by H&E staining (Fig. 2e, f). In Fig. 3, we show some examples of the diversity of IIF staining on monkey esophagus seen in patients affected by EPF in El Bagre in order to illustrate the differences between the IIF staining of this El Bagre EPF variant and other pemphigus. Figure 3a–d displays the autoreactivity seen in classical ICS of pemphigus, but, as seen in Senear-Usher syndrome, we also see the presence of lupus band-like staining at the BMZ. We also show some intracytoplasmic and perinuclear staining whose specific antigens still need to be uncovered. In Fig. 3c and d, we show autoreactivity to some superficial skin vessels located under the BMZ, as well as some reactivity to part of the *muscularis* of the mucosa of the monkey esophagus samples. These results illustrate the great diversity of autoreactivity seen in affected individuals by this unique disease. Finally, Fig. 3 shows representative IB results of several El Bagre EPF cases and controls, illustrating strong reactivity to plakins and many other unidentified antigens.

Table 1 Summarize the most common patterns of axillary lesions and immunopathological findings in the case El Bagre EPF cases *versus* the controls.

Discussion

We were able to demonstrate that patients affected by Bagre-EPF have very unique immunological and clinical manifestations in comparison with other described forms of pemphigus. In this study, we determined that several patients affected by El Bagre-EPF have axillae lesions, and a wide immune response is seen against sweat glands as determined by DIF, IIF and IHC. These data, together with our previous description of the presence of mercuric selenide and sulfides within their sweat glands, led us to investigate whether the autoreactivity we find in these studies may be part of this complex disease. We also found that, in

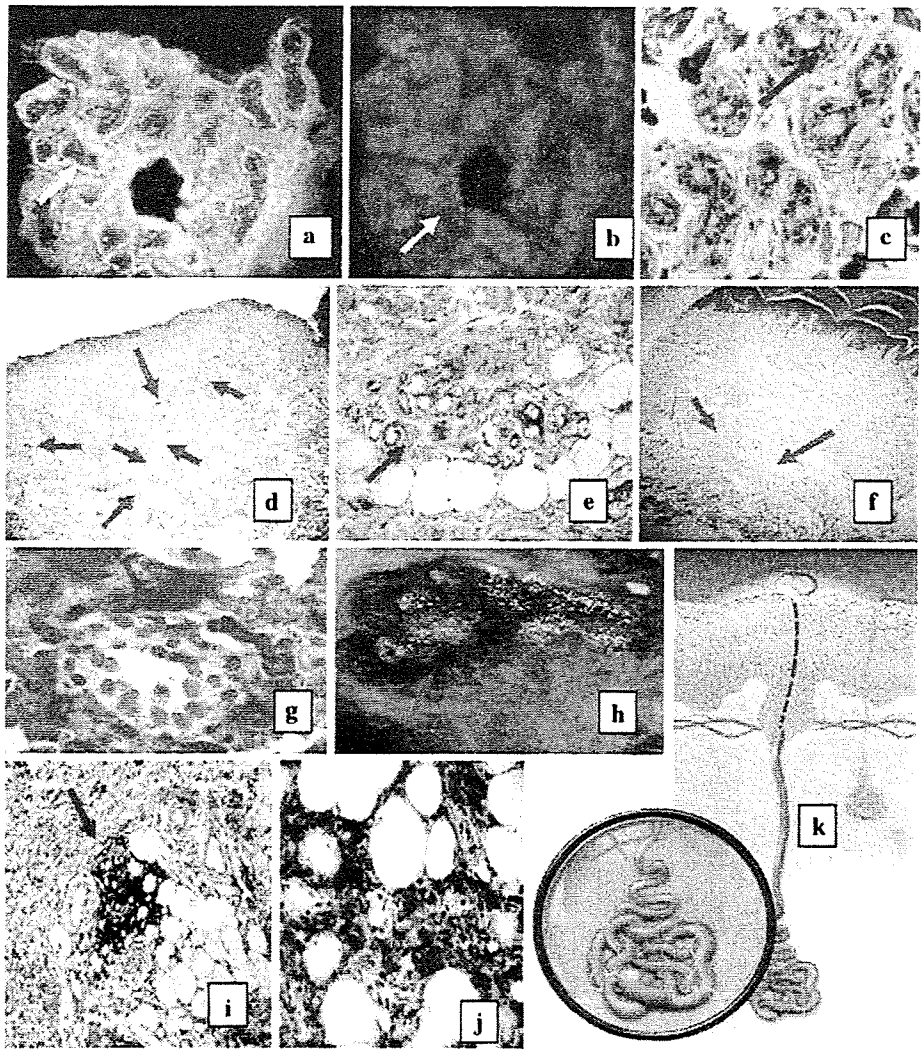
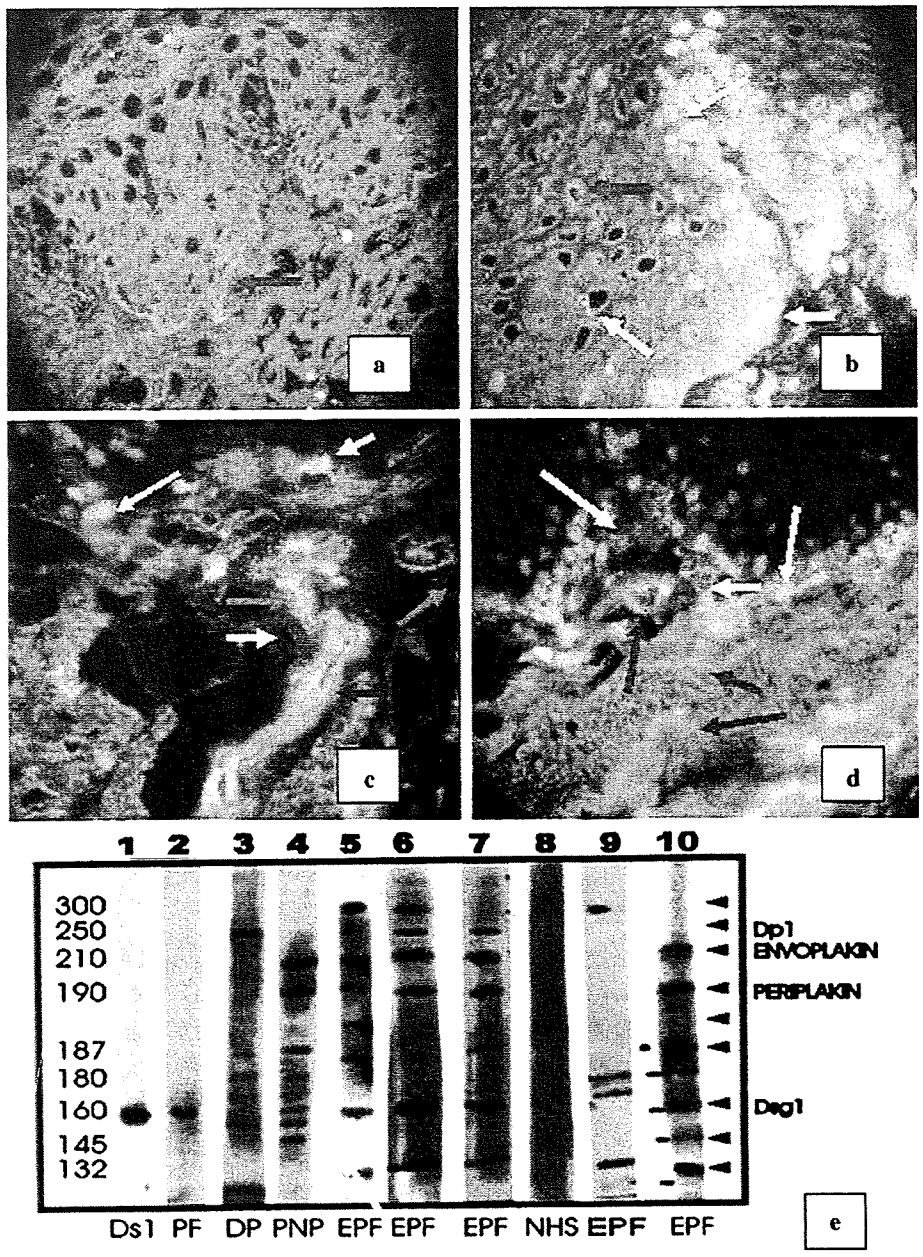


Fig. 2 **a** and **b** IIF shows a group of positive reactivity to sweat glands when using anti-human-fibrinogen FITC-conjugated antibodies (×40). Note the increase of fluorescence around each intraglandular coiled duct, as well as in the coiled glandular globule glands (*green*-fluorescence staining) (*white arrow*). In this case the nuclei were counterstained with TOPRO-3® (*red dots*). **b** We repeated the experiments using unconjugated rabbit anti-human fibrinogen antibodies, and as a secondary we used rabbit anti-human Alexa 555 (both from Invitrogen). In this case, **b** shows a strong positive staining with fibrinogen (red staining) (*yellow arrow*). **c**, **d**, **j**, and **i** We performed IHC studies, to avoid autofluorescence of the sweat glands and to confirm a specific immune response to these glands. For this reason, we decided to use a non-fluorescent method to probe real reactivity versus non-specific reactivity using HRP. In Fig. 2c, we were able to demonstrate a positive *brownish* staining in the sweat glands using CEA (*red arrow*). In **g**, we stained with anti-CD3 (dark stain) (*red arrow*), and in **i** with anti-CD68 (dark staining) (*red arrow*). Finally, in **j**, IHC was performed on a paraffin-fixed skin sample from the axillae of one El Bagre EPF patient. We used mouse anti-human CD20 monoclonal antibody following the manufacturer's instruction. We were able to visualize CD20 positive cells around the sweat glands of this patient (×40), indirectly showing a specific immune response (*yellow arrow*) (*brownish* staining). **h** IIF staining utilizing mouse-anti-human IgA-conjugated with FITC (*white arrow*) (a *yellowish* staining) can also be due to a non-specific reaction due to the visualization of both lipofuscin granules and secretory IgA (*blue arrow*). **k** A schematic representation of each gland, which consists of a secretory component and an excretory duct. The secretory component is located immediately below the dermis in the subcutaneous tissue and is coiled upon itself. The secretory portion of the gland changes into a duct that proceeds toward the surface. The duct follows a spiral course through the dermis (reproduced with permission of the Skin Care Forum)

chronic cases, several skin appendices, including the sweat glands, were lost in patients affected by El Bagre EPF as visualized by H&E. In conjunction with this, sclerodermoid changes, or early sclerodermatous changes, sometimes extended into the adipose tissue as a membranous lipodystrophy. Our data prompt us to believe that this immunoreactivity toward sweat glands in El Bagre EPF patients may destroy several of these structures in several chronic cases. In addition to the reactivity to the sweat glands, we have found autoreactivity to neuro-vascular



plexus that feed the sweat glands, the sebaceous glands and other structures on the skin. (Abreu et al., manuscript in preparation). Based on these recent discoveries we cannot say yet what is the primary phenomenon (the reactivity to the sweat glands and/or to the neurovascular package) that feed this and other skin appendices. We found the dilemma of determining what was first—the chicken or the egg?

In regards to whether the eccrine or apoeccrine glands showed autoreactivity in our studies, with our current findings, we can only indirectly deduce that eccrine glands are affected, based on their location and microscopical features [7, 10, 12, 18]. However, further studies using skin from genital, peri-oral, or peri-areolar areas that are enriched with apoeccrine glands need to be performed. Traditionally, the most well-known important physiological function of eccrine glands is sweat production and cooling; however, given the physiological role and extensive and complex presence of multiple molecules, the role of these glands may be more complex. Some studies have shown that these glands could play a role in metabolism, hormone balance, adsorption, and possibly immune regulation. With this study we demonstrated autoantibodies to several components of the sweat glands. The IIF titers indicate that this phenomenon is not the result from non-specific adherence

◀ **Fig. 3** **a** and **b** IIF using monkey esophagus (Primate Center Portland, Oregon) as an antigen source for testing one of the El Bagre EPF serum samples using FITC-conjugated anti-human IgM antibodies. **a** Some perinuclear and intracytoplasmic staining inside the keratinocytes is seen (*red arrows*) (×20). **b** Similar to **a** but with DAPI as a nuclear counterstain (*blue* stain) (*white arrows*), which shows more clearly the intracellular staining (*red arrows*). In this case we used anti-collagen IV (CIV) monoclonal antibody and Alexa 555 as a secondary antibody (*red* staining). There CIV positive staining at the BMZ and a little is also present among some keratinocytes (*yellow arrows*). **c** we show autoreactivity to some superficial and deep skin vessels located under the BMZ using anti-human fibrinogen-FITC conjugated antibody (*green* stain) (*blue arrows*). The *red-orange dots* staining shows colocalization of the patient's antibodies with an antibody to CIV around those vessels. The nuclei were counterstained with Dapi (*blue* stain) (*white arrow*) **d** IIF of monkey esophagus using an El Bagre-EPF patient serum sample with FITC-conjugated anti-human fibrinogen antibody at higher amplification (×100). Staining shows a positive lupus-band like staining at the BMZ (*greenish* staining) (*fuchsia arrows*). The *red arrows* point to a high positivity on the muscles of the mucosa using the same antibody (*greenish* staining). The *blue arrow* shows a very positive blood vessel below the BMZ (*greenish* staining). The nuclei were counterstained with DAPI (*blue* stain) (*white arrow*). Positive reactivity occurred with collagen IV (CIV) antibody in clumps around the BMZ (*green arrow*) (*reddish* staining) ×40. **e** IB performed using ECL. *Lane 1* is Dsg1 control (Progen, Heidelberg); *lane 2* is serum from a sporadic PF patient with a positive band of 160 kDa (Dsg1); *lane 3* is desmoplakin antibody from Progen (the main band is 250 kDa, but smaller possible degradation products as the result of the skin antigen preparation are seen); *lane 4* shows the autoreactivity from a PNP serum showing positivity to envoplakin, periplakin, and Dsg1 with other bands; *lanes 5, 6, 7, 9* and *10* are several sera from El Bagre EPF patients recognizing several plakins, desmoglein molecules and some others of unknown identity. Note that serum 10 shows some variability in the immune response. *Lane 8* is a normal human serum (NHS) control, which is negative. The molecular weight standards are shown in the left

of antibodies, indeed the presence of CD3, CD20 and CD68 shows an ongoing specific antigen presentation and selective immune response.

In our studies, we addressed the intrinsic fluorescence of the lipofuscin granules and secretory IgA by utilizing IHC to determine whether the reactivity is truly sweat gland immunoreactivity or the background autofluorescence of sweat glands. Our results confirmed a specific immunological response. For interpretation of our studies using IIF and DIF, we also took into consideration previously documented capability of some sweat glands to directly absorb selected dyes such as Bodipy FL C_5. We avoided using this fluorophore for our experiments [15, 23]. Previous reports on the autofluorescence of sweat glands documented diffusion of Bodipy FL C_5 into the sweat glands after 4–5 h and in the fat cells surrounding the sweat glands [15, 23]. In light of our findings, it is important to take into consideration that not only El Bagre EPF patients but also three patients with PNP showed autoreactivity to adnexal structures, including the sweat glands [6]. The authors have suggested that desmoplakins can be more strongly expressed in adnexa, including the sweat glands, than in the epidermis, facilitating visualization of antibody deposits [6]. El Bagre-EPF patients have several autoantibodies shared with patients suffering from PNP, as shown in this study and as previously reported by us and others [2–5]. Other studies have shown that patients were affected by cicatricial pemphigoid (CP) and linear deposits of IgG, C3 and fibrinogen in the base membranes of sweat glands ductus [9, 13, 21, 22].

Several pathological alterations have been documented in sweat glands, including electrolyte reabsorption, as shown in eccrine pseudochromohidrosis, where blue sweat presents in copper workers [14]. Other toxicological and metabolic alterations have been documented for these glands, including their role in patients with generalized argyria, with low ceruloplasmin and copper levels in the serum during a trial of penicillamine treatment [14]. The sweat glands also play an important role in ion exchange processes, including methylcholine-induced sweat secretion dependent on extracellular and/or the intracellular calcium concentrations [20]. Metals, metalloids and hormones

Table 1 Summarize the most common patterns of axillary lesions and immunopathological findings in the case El Bagre EPF cases versus the controls

Immunopathological findings	El Bagre-EPF	Controls from endemic an non-endemic area
Positive response by IHC using CD3, CD68, and CD20,	7/10 (70%)	None
DIF of the sweat glands acrosyringium using IgG, albumin, fibrinogen or C3 FITC conjugated antibodies	8/10 (100%)	Albumin (+/−), fibrinogen (+/−) in 1/10 controls from the endemic area, and negative in 10/10 controls from the non endemic area
DIF positive at the coiled portion using IgG, albumin, fibrinogen or C3 FITC conjugated antibodies	5/5 (100%)	Negative
IIF positive at the coiled portion using IgG, albumin, fibrinogen or C3 FITC conjugated antibodies	8/10 (80%)	None
Necrosis of sweat glands by H & E	3/10 (30%)	None
Peri-inflammatory infiltrate around all the sweat glands with a predominance of lymphohistocytes by H & E	8/10 (80%)	None

can interact with and compete in an allosteric manner with Ca^{++}. Of importance to our findings is the fact that several other autoimmune skin diseases have been shown to have autoreactivity to sweat glands, including individuals with systemic lupus erythematosus, Sjögren's syndrome, systemic sclerosis/morphea, diabetes, active acute systemic multiple sclerosis, and sudden onset juvenile idiopathic arthritis [16, 17].

Finally, as an associated or completely coincidental finding, the presence of eccrine syringofibroadenomatosis in two patients with bullous pemphigoid and acantholysis within the sweat glands in Grover's syndrome have been described [11, 19]. Taken together, these compiled findings lead to questions regarding complex role of sweat glands in El Bagre-EPF.

Acknowledgments Georgia Dermatopathology Associates (MSH), Atlanta, GA, USA. The El Bagre-EPF samples were collected from previous grants from the Embassy of Japan in Colombia, DSSA, U. de A. and Mineros de Antioquia SA, (AMAV), Medellin, Colombia, South America.

Conflict of interest statement None.

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A randomized double-blind trial of intravenous immunoglobulin for pemphigus

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Tokyo, Sapporo, Asahikawa, Hirosaki, Sendai, Fukushima, Kawagoe, Yokohama, Hamamatsu, Ogaki, Ohtsu, Kyoto, Suita, Osaka, Kashihara, Wakayama, Okayama, Hiroshima, Kitakyusyu, Fukuoka, Kurume, Kumamoto, Yufu, Gifu, and Toon, Japan

Background: Pemphigus is a rare life-threatening intractable autoimmune blistering disease caused by IgG autoantibodies to desmogleins. It has been difficult to conduct a double-blind clinical study for pemphigus partly because, in a placebo group, appropriate treatment often must be provided when the disease flares.

Objective: A multicenter, randomized, placebo-controlled, double-blind trial was conducted to investigate the therapeutic effect of a single cycle of high-dose intravenous immunoglobulin (400, 200, or 0 mg/kg/d) administered over 5 consecutive days in patients relatively resistant to systemic steroids.

Methods: We evaluated efficacy with time to escape from the protocol as a novel primary end point, and pemphigus activity score, antidesmoglein enzyme-linked immunosorbent assay scores, and safety as secondary end points.

Results: We enrolled 61 patients with pemphigus vulgaris or pemphigus foliaceus who did not respond to prednisolone (≥20 mg/d). Time to escape from the protocol was significantly prolonged in the 400-mg group compared with the placebo group ($P < .001$), and a dose-response relationship among the 3 treatment groups was observed ($P < .001$). Disease activity and enzyme-linked immunosorbent assay scores were significantly lower in the 400-mg group than in the other groups ($P < .05$ on day 43, $P < .01$ on day 85). There was no significant difference in the safety end point among the 3 treatment groups.

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Other investigators in the Pemphigus Study Group are listed in the Appendix.

Supported by Nihon Pharmaceutical Co Ltd, but no financial support was provided to any individual investigator for performing this trial.

Disclosure: Drs Amagai, Ikeda, Kitajima, Nishikawa, and Hashimoto report receiving consulting and lecture fees from Nihon Pharmaceutical Co Ltd. Drs Shimizu, Iizuka, Hanada, Aiba, Kaneko, Izaki, Tamaki, Ikezawa, Takigawa, Seishima, Tanaka, Miyachi, Katayama, Horiguchi, Miyagawa, Furukawa, Iwatsuki, Hide, Tokura, Furue, Ihn, Fujiwara, Ogawa, and Hashimoto have no conflicts of interest to declare.

Presented in part at the Post International Investigative Dermatology Satellite International Meeting on Autoimmune Bullous Diseases at Ohtsu, Japan on May 19, 2008 and the Annual Meeting of the Japanese Dermatological Association at Kyoto, Japan on April 20, 2008.

Accepted for publication September 28, 2008.

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0190-9622/$36.00

doi:10.1016/j.jaad.2008.09.052

Limitation: Prednisolone at 20 mg/d or more may not be high enough to define steroid resistance.

Conclusion: Intravenous immunoglobulin (400 mg/kg/d for 5 d) in a single cycle is an effective and safe treatment for patients with pemphigus who are relatively resistant to systemic steroids. Time to escape from the protocol is a useful indicator for evaluation in randomized, placebo-controlled, double-blind studies of rare and serious diseases. (J Am Acad Dermatol 2009;60:595-603.)

Pemphigus is a life-threatening, rare intractable autoimmune blistering disease caused by IgG autoantibodies to desmoglein (Dsg) (epidermal adhesion factor). It is characterized by the development of blisters and erosions of the skin and mucosa.[1] Currently, oral steroids are the drugs of first choice for pemphigus, and may be used in combination with immunosuppressants or plasma exchange. However, many patients with pemphigus experience cycles of remission and recurrence, and accordingly become unresponsive to conventional therapy. On the other hand, patients with complications such as diabetes mellitus, gastrointestinal disease, osteoporosis, infection, or immunodeficiency are relatively contraindicated for use of high-dose (HD) steroids. For such patients, an alternative effective treatment strategy is required.

Although several reports suggesting the effectiveness of HD intravenous immunoglobulin (IVIG) in the treatment of pemphigus have been published since its introduction as monotherapy in 1989, most are case reports with a low evidence level or involved clinical research with a limited number of patients using multiple treatment cycles.[2] No well-controlled, double-blind clinical study to demonstrate the efficacy of HD-IVIG has been conducted.[3-13] This is because: (1) pemphigus is a rare intractable disease; (2) appropriate treatment must be provided in a timely manner if symptoms are aggravated or unchanged for a certain period of time; (3) inclusion of a placebo group compromises compliance with the study protocol; and (4) it is not ethical to treat patients with pemphigus using placebo because mortality is high.

We developed a novel evaluation end point to solve these problems and verified the usefulness of HD-IVIG in a single treatment cycle for this rare intractable disease.

Abbreviations used:

ADRs:	adverse drug reactions
Dsg:	desmoglein
HD:	high dose
IVIG:	intravenous immunoglobulin
PAS:	pemphigus activity score
PF:	pemphigus foliaceus
PV:	pemphigus vulgaris
TEP:	time to escape from the protocol

METHODS

Patients

This study was conducted in 27 medical institutions in Japan with affiliated dermatologists specialized in autoimmune blistering disease. Patients were given the diagnosis of pemphigus vulgaris (PV) or pemphigus foliaceus (PF) as confirmed based on our national diagnostic criteria as follows: pemphigus was diagnosed when at least one item from every 3 findings, or two items from clinical findings and one item from immunologic findings were satisfied.

1. Clinical findings
   - Multiple, easily rupturing, flaccid blisters of the skin
   - Subsequent progressive, refractory erosions or crust after blisters
   - Noninfectious blisters or erosions of visible mucosa including oral mucosa
   - Nikolsky sign
2. Histologic findings
   - Intraepidermal blisters caused by loss of adhesion between epidermal cells (acantholysis)
3. Immunologic findings
   - IgG (or complement) deposition in the intercellular spaces of the lesional or normal-appearing skin and mucosa as detected by direct fluorescent antibody assay
   - Antiepidermal intercellular IgG autoantibody (anti-Dsg IgG autoantibody) identified by indirect fluorescent antibody assay or enzyme-linked immunosorbent assay

The study patients had to meet all the following inclusion criteria and none of the exclusion criteria.

1. Inclusion criteria: patients aged 20 years or older who provided written informed consent to participate in the study and met all of the following criteria.

Table I. Criteria for pemphigus activity score

Variable score	Skin lesion area*	No. of new blisters/d	Oral mucosal lesions†
3	≥15%	≥5	≥30%
2	≥5% and <15%	1 to 4	≥5% and <30%
1	<5%	Occasionally‡	<5%
0	None	None	None

*Percentage of entire surface area.
†Score is doubled for patients who have only oral mucosal lesions at time of study enrollment.
‡Blisters sometimes newly develop within 1 week but not every day.

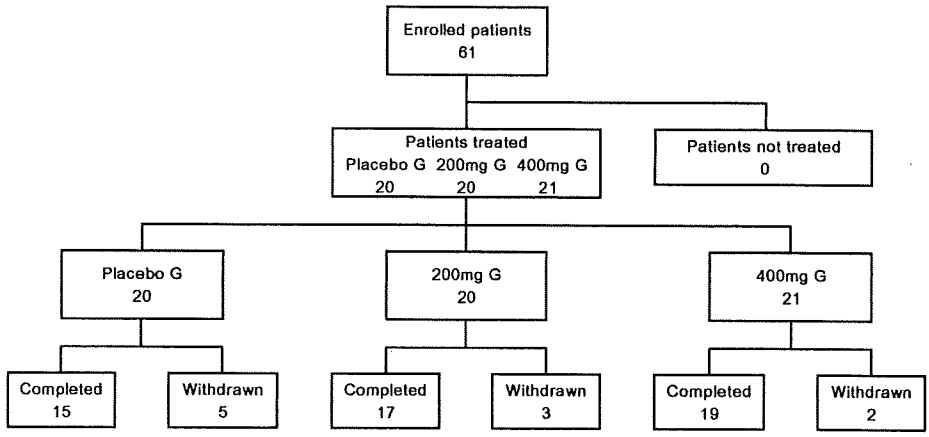

Fig 1. Disposition of patients. *G*, Group.

- Treatment with any steroid at greater than or equal to 20 mg/d (prednisolone equivalent)
- Symptoms (total pemphigus activity score [PAS] [Table I]) did not respond to steroid therapy

2. Exclusion criteria: patients who met any of the following criteria were excluded from the study because efficacy evaluation of the test drug might be affected and to assure the safety of patients.
   - Patients treated with plasma exchange therapy, steroid pulse therapy, or HD-IVIG within 30, 14, or 42 days, respectively, before informed consent and the start of study treatment
   - Patients with a history of shock or hypersensitivity to the test drug
   - Patients with IgA deficiency, hepatic disorder, renal disorder, or hemolytic or blood loss anemia
   - Patients with any previous or existing cerebrovascular or cardiovascular disorder

Study design

This was a multicenter, randomized, placebo-controlled, double-blind, parallel-group study. The study protocol and written informed consent form approved by the institutional review board at each study institution were used in the study. Observation of the first patient was started on November 4, 2004, and that of the last patient was completed on September 25, 2006.

Treatment groups

The IVIG group received IV drip infusion at 200 or 400 mg/kg/d administered in divided dose over 5 consecutive days. The placebo group received IV drip infusion of physiologic saline for 5 consecutive days.

Investigational drugs manufactured by Nihon Pharmaceutical Co Ltd (Higashikanda, Tokyo, Japan) were used in the study.

Methods of allocation

Patients were randomized by a central enrollment system to the treatment groups according to a dynamic allocation scheme to ensure that there were no between-group differences in the dose of prior steroid, total PAS, or disease type.

Blinding

Because the investigational drugs were distinguishable in terms of appearance and viscosity after

Table II. Demographic and other baseline characteristics

Characteristic	Category	Dose: Placebo n = 20	Dose: 200 mg n = 20	Dose: 400 mg n = 21	Between-group comparison
Sex	Male	9	10	8	NS* (*P* = .766)
	Female	11	10	13	
Age, y	Mean ± SD	53.1 ± 10.9	57.0 ± 14.6	50.1 ± 11.7	NS† (*P* = .225)
Body weight, kg	Mean ± SD	57.8 ± 11.6	58.0 ± 10.4	57.7 ± 9.1	NS* (*P* = .686)
Disease type	PV	13	14	13	NS* (*P* = .942)
	PF	7	6	8	
Disease duration, mo	Mean ± SD	16.1 ± 13.6	28.6 ± 32.3	28.5 ± 46.9	NS† (*P* = .414)
Baseline PAS	Mean ± SD	3.3 ± 1.4	3.6 ± 1.8	3.7 ± 1.1	NS† (*P* = .660)
Steroid dose, mg	Mean ± SD	27.6 ± 9.7	23.9 ± 11.1	27.4 ± 11.1	NS† (*P* = .461)
Immunosuppressants	No. of patients (%)	2 (10.0)	7 (35.0)	5 (23.8)	NS* (*P* = .179)

NS, Not significant difference; *PAS*, pemphigus activity score; *PF*, pemphigus foliaceus; *PV*, pemphigus vulgaris.
Two-sided test for both analyses.
*Fisher exact test.
†One-way analysis of variance.

reconstitution, independent staff at each study institution separately prepared and administered the dosing solution, and evaluated efficacy and safety in each patient to maintain blinding. The bottles of the investigational drugs were covered with a masking cover and provided to the independent staff member in charge of administration. Each independent staff member involved signed a blinding confirmation form at the end of the study to assure that blinding was maintained.

End points

Time to escape from the protocol (TEP) was used as the primary efficacy end point. TEP was defined as the length of the period until a patient stayed on the protocol without any additional treatment. When symptoms were unchanged for 2 weeks or aggravated, the treatment given was considered to be ineffective and additional treatment was required such as increase in steroid dose, change in steroid type, use of additional immunosuppressive agents, or plasma exchange; these patients were considered escaped from the protocol. This methods allow doctors in charge to have flexibility to rescue patients with other treatment when needed.

The secondary end points used in the study included: (1) PAS over time (scores [0-3 point] for skin lesion area, number of new blisters/d, and oral mucosal lesions, and their total scores [Table I]); and (2) the titers of pemphigus autoantibodies over time (anti-Dsg1 autoantibody titer and anti-Dsg3 autoantibody titer). Titers of pemphigus autoantibodies were determined by enzyme-linked immunosorbent assay.[14,15] As a safety end point, the occurrence of adverse events by 85 days after the start of the study treatment (day 85) was investigated. Adverse events were recorded up to day 43 if patients escaped from the protocol by day 43 or up to TEP if patients escaped from the protocol after day 44.

Statistical analysis

The cumulative rate of TEP, which was estimated by evaluation of the dose-response relationship of TEP and by analysis using the Kaplan-Meier method, was compared among the treatment groups by log rank test. Scores for skin lesion area, number of new blisters/d, and oral mucosal lesions, and total score, the secondary end point, up to day 85 were compared with baseline data by the paired *t* test for each treatment group. The data after TEP were imputed from the data at the TEP (last observation carried forward). Adverse events occurring up to day 85 for which the causal relationship with HD-IVIG or placebo was judged to be other than "not related" were handled as adverse drug reactions (ADRs). A two-sided significance level of .05 was used for analyses.

RESULTS

Disposition of patients

The disposition of patients enrolled in the study is shown in Fig 1. A total of 61 patients were treated with the investigational drug (placebo, 20; 200 mg, 20; and 400 mg, 21). All the enrolled patients including 10 patients (placebo, 5; 200 mg, 3; and 400 mg, 2) who were withdrawn from the study according to the requirements in the protocol were included in the analyses. The main reasons for study withdrawal were the evaluator's decision to withdraw the patient and the occurrence of adverse events. The demographic and other baseline

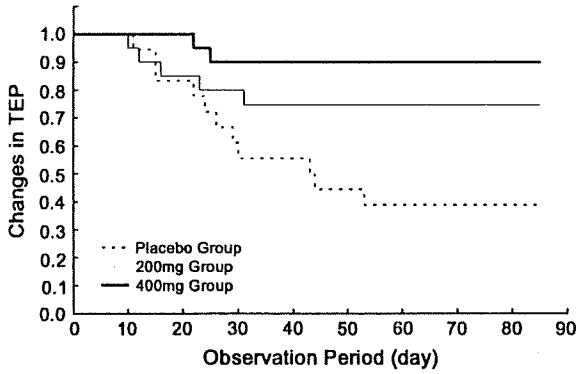

Fig 2. Changes in time to escape from protocol (*TEP*). TEP was significantly prolonged in 400-mg group compared with placebo group with dose-dependent fashion. Cumulative TEP on day 85 was 10.0% in 400-mg group, 25.0% in 200-mg group, and 61.0% in placebo group (log rank test). Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group ($P < .001$, log rank test). In contrast, difference between 200-mg and placebo groups was not significant ($P = .052$). In addition, dose-response relationship was observed in TEP ($P < .001$). Data are stated using TEP ratio.

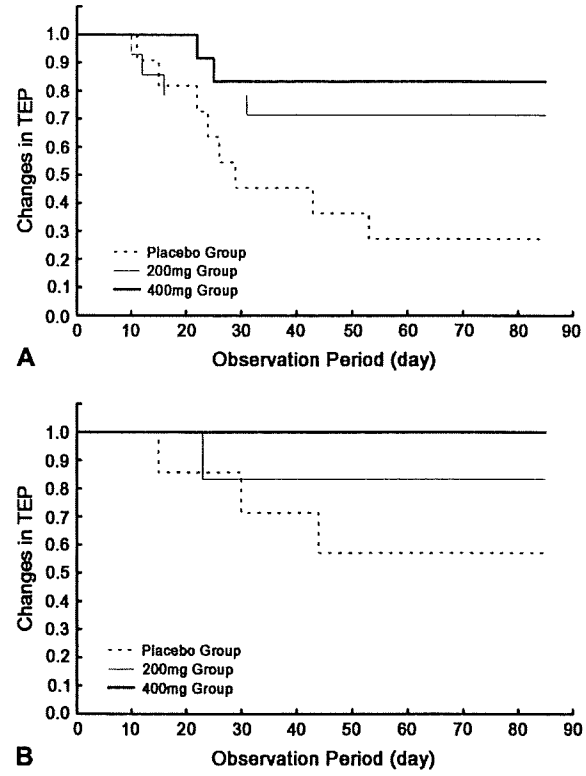

Fig 3. Cumulative time to escape from protocol (*TEP*) shown by pemphigus subtype. Cumulative TEP estimated by Kaplan-Meier method was divided in disease subtype of pemphigus vulgaris (PV) (**A**, n = 13 in 400-mg group, n = 14 in 200-mg group, n = 13 in placebo group) and pemphigus foliaceus (PF) (**B**, n = 8 in 400-mg group, n = 6 in 200-mg group, n = 7 in placebo group). Cumulative TEP in patients with PV on day 85 was 15.0% in 400-mg group, 29.0% in 200-mg group, and 73.0% in placebo group, whereas that of patients with PF was 0.0% in 400-mg group, 17.0% in 200-mg group, and 43.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (PV, $P = .007$; PF, $P = .044$; log rank test). In contrast, difference between 200-mg and placebo groups was not significant (PV, $P = .055$; PF, $P = .416$). In addition, dose-response relationship was observed in TEP (PV, $P = .007$; PF, $P = .043$).

characteristics are presented in Table II. There were no significant between-group differences in the distribution of baseline characteristics. The average disease durations of 200- and 400-mg groups are longer than in the placebo group, but this is because the former group happened to contain patients with extremely long duration (116 months in 200 mg; 142 and 169 months in 400 mg) and the difference was not statistically significant.

Efficacy (primary end point): TEP

TEP was evaluated as the primary end point (Fig 2). In the 400-mg group, 19 of 21 patients stayed on the protocol during the observation period. Two patients escaped from the protocol with TEPs of 22 and 25 days. In the 200-mg group, 15 of 20 patients stayed on the protocol and the shortest TEP was as early as 10 days among the 5 escaped patients. In the placebo group, only 9 patients stayed on the protocol, and the shortest TEP was as early as 11 days. TEP was within 30 days for 8 patients.

TEP in the active treatment groups was compared with that in the placebo group (log rank test). The TEP in the 400-mg group was significantly longer than that in the placebo group ($P < .001$), whereas the difference between the 200-mg and placebo groups was not significant ($P = .052$). Log rank test of TEP for the 61 patients indicated a dose-response relationship for this parameter ($P < .001$).

Analyses stratified by baseline characteristics (disease type and PAS) also demonstrated dose-response relationships and significant differences between the 400-mg and placebo groups, as in the overall analyses (Figs 3 and 4).

Efficacy (secondary end point)

Pemphigus activity score. Efficacy was also evaluated based on the changes in clinical symptoms, ie, changes in PAS determined based on skin lesion area, number of new blisters/d, and oral mucosal lesions. In the 400-mg group, total PAS

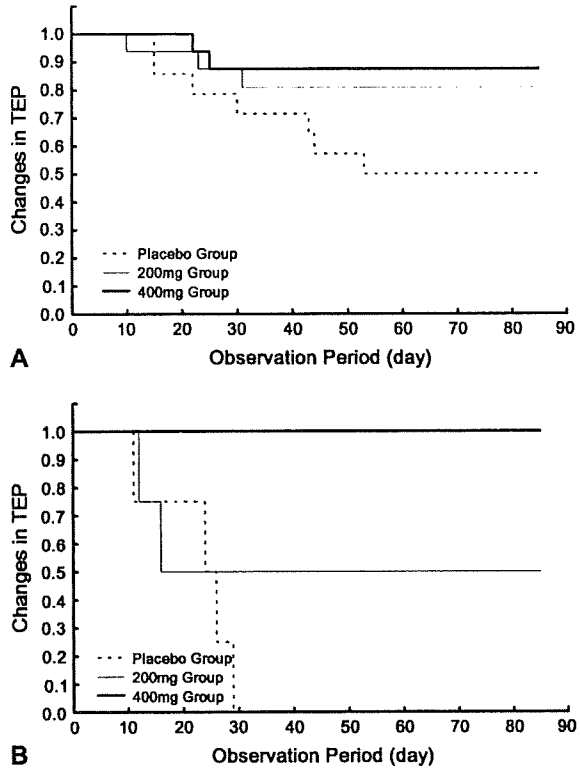

Fig 4. Cumulative time to escape from protocol (*TEP*) shown in different pemphigus activity score (PAS). Data were divided by PAS into two groups: total PAS of 0 to 4 (**A**, n = 17 in 400-mg group, n = 16 in 200-mg group, n = 16 in placebo group) and total PAS of 5 to 9 (**B**, n = 4 in 400-mg group, n = 4 in 200-mg group, n = 4 in placebo group). Cumulative TEP in patients with total PAS of 0 to 4 on day 85 was 12.0% in 400-mg group, 19.0% in 200-mg group, and 50.0% in placebo group, whereas those of patients with total PAS of 5 to 9 was 0.0% in 400-mg group, 50.0% in 200-mg group, and 100.0% in placebo group. Between-group comparison demonstrated significant prolongation of TEP in 400-mg group compared with placebo group (total score 0-4, $P = .028$; total score 5-9, $P = .006$). In contrast, difference between 200-mg and placebo groups was not significant (total score 0-4, $P = .109$; total score 5-9, $P = .345$). In addition, dose-response relationship was observed in TEP (total score 0-4, $P = .024$; total score 5-9, $P = .012$).

was significantly decreased from the baseline score at all points of observation (day 8, $P = .05$; after day 15, $P < .01$). It was decreased from 3.7 on day 1 to 2.0 on day 85 (by 46.8%) (Fig 5). In the 200-mg group, total PAS was significantly decreased from the baseline score at all points of observation after day 15 (day 15-43, $P < .05$; day 57-85, $P < .01$). It was decreased from 3.7 on day 1 to 2.3 on day 85 (by 36.6%). On the other hand, in the placebo group, no significant decrease from baseline score was observed at any of the points of observation. Each PAS

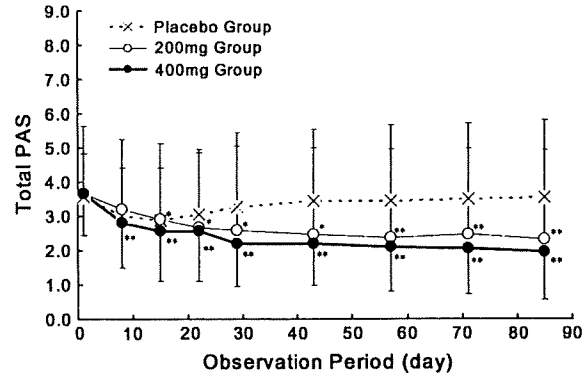

Fig 5. Changes of pemphigus activity score (*PAS*) over time. Total PAS was significantly lower in 400- and 200-mg groups than in placebo group. Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

(skin lesion area, number of new blisters/d, and oral mucosal lesions) also exhibited a significant change from baseline in the 400-mg group ($P < .01$) but not in the placebo group (data not shown).

Titers of anti-Dsg IgG autoantibodies

It has been reported that levels of IgG autoantibodies to Dsg1 and Dsg3 in patients with pemphigus correlate with disease activity.[14,15] Accordingly, efficacy was also evaluated based on the changes in anti-Dsg1 IgG autoantibody titer for patients with PF and PV or in anti-Dsg3 IgG autoantibody titer for patients with PV (Fig 6). In the 400-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers were significantly decreased from baseline on days 43 and 85 (day 43 and 85, $P < .01$). In the 200-mg group, anti-Dsg1 and −Dsg3 IgG antibody titers also exhibited significant decreases on day 85 but not day 43 (day 43, $P < .05$; day 85, $P < .01$). On the other hand, in the placebo group, no significant decrease from baseline was observed in either anti-Dsg1 or −Dsg3 IgG antibody titer.

Safety

The incidence of ADRs was 28.6% (n = 6/21) in the 400-mg group, 35.0% (n = 7/20) in the 200-mg group, and 25.0% (n = 5/20) in the placebo group. No significant difference was observed between the placebo and 200- or 400-mg groups. ADRs reported in the study included: headache in two patients, aggravated chronic hepatitis C, decreased lymphocytes, palpitations, abdominal discomfort, constipation, nausea, pain at the injection site, increased creatinine, increased blood pressure, and decreased platelet count in one patient each in the 400-mg group; and increased alanine aminotransferase in 3 patients; increased γ-glutamyltranspeptitase, hepatic dysfunction, and increased bilirubin in two patients each; and common cold, muscle pain, increased

aspartate aminotransferase, increased blood pressure, decreased lymphocytes, increased neutrophils, decreased white blood cell count, bleeding tendency, anorexia, hypoalbuminemia, hepatic encephalopathy, gastrointestinal bleeding, malaise, fever, increased ammonium, increased C-reactive protein, decreased hematocrit, decreased hemoglobin, decreased platelet count, decreased red blood cell count, and decreased urine volume in one patient each in the 200-mg group. All these ADRs were consistent with the information displayed on the Food and Drug Administration Web site (http://www.fda.gov/cber/gdlns/igivimmuno.htm).

One patient in the 200-mg group died of hepatic failure as a result of aggravation of hepatitis C, which was an underlying complication reported before the start of the study.

This event was judged as probably related to the investigational drug in the evaluator's opinion.

DISCUSSION

Most clinical research involving a rare disease is based on case reports or data from limited samples obtained in open-label studies. In particular, in life-threatening, serious, and intractable diseases, such as pemphigus, appropriate treatment must be provided in a timely fashion if symptoms are aggravated or unchanged for days. This makes performance of a placebo-controlled, double-blind comparison study infeasible. On the other hand, the efficacy of new drugs for malignant tumors or for patients requiring pain relief is evaluated based on the time to recurrence of tumor or the number of patients requiring rescue analgesia.[16-21] Based on these considerations, we developed a novel efficacy indicator (ie, TEP) with reference to the end points used for efficacy evaluation of drugs for malignant tumors or for patients requiring pain relief, to conduct a placebo-controlled, double-blind comparison study in patients with pemphigus who were relatively resistant to systemic steroids. This new efficacy end point provides flexibility for physicians to rescue patients when required and proved to be useful to evaluate the efficacy of a single cycle of HD-IVIG in a double-blind comparison design. However, some concerns remain regarding the rigidity: a period of 3 to 7 days before the start of study treatment was required to confirm the unresponsiveness of patients to steroids, and switching to other treatments was prohibited during the first 5-day treatment period.

The mode of action of HD-IVIG is complex. It is found to exert its effect through modulation of expression and function of Fc receptors, interference with complement activation and the cytokine

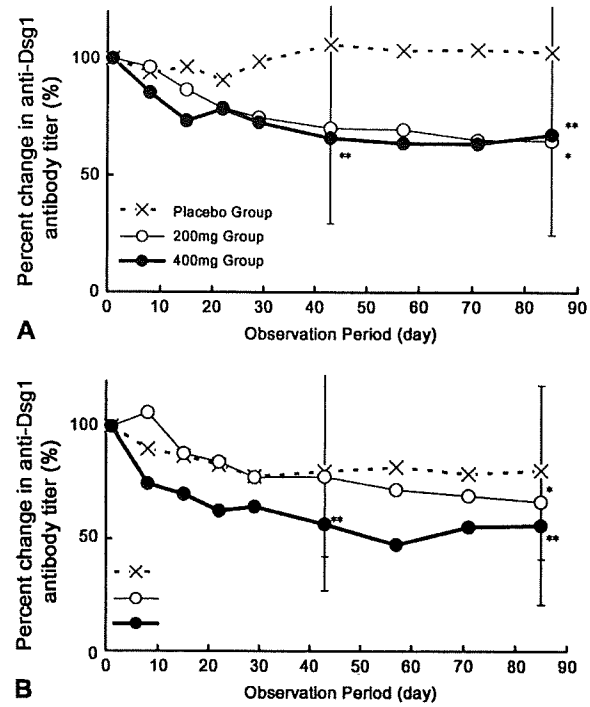

Fig 6. Changes of anti-desmoglein (*Dsg*) IgG titers. Anti-Dsg IgG titers were significantly lower in 400-mg intravenous immunoglobulin group than in placebo group over time. Changes of titers in anti-Dsg1 IgG autoantibodies (**A**) in patients with pemphigus vulgaris (PV) and pemphigus foliaceus and in anti-Dsg3 IgG autoantibodies (**B**) in patients with PV were shown (mean ± SD). Significant difference from day 1 at hazard ratio of *0.05 and **0.01.

network, provision of anti-idiotypic antibodies, modulation of dendritic cell, T- and B-cell activation, differentiation, and their effector functions.[22,23] Thus, HD-IVIG has multiple modes of action and is thought to act synergistically. HD-IVIG exerts immunomodulatory effects in autoimmune and inflammatory disorders without suppressing the immune system, which provides a distinctive advantage over conventional treatment.

Most of the previous studies suggesting efficacy of HD-IVIG for treatment of pemphigus involved multiple treatment cycles. However, our study demonstrated that a single cycle with HD-IVIG for 5 days has a therapeutic benefit to suppress the disease activity of pemphigus. Like rituximab, for which efficacy was recently reported in a single cycle,[24] IVIG is expensive and should be considered for patients who show difficulty with or resistance to conventional treatments.

In conclusion, our study suggests that TEP is a useful indicator for evaluation for rare intractable diseases such as pemphigus, and that a single cycle of HD-IVIG appears to be an effective treatment for

patients with pemphigus who are relatively resistant to systemic steroids.

We thank members of the Pemphigus Study Group described below for cooperation with registration and precise observation of patients with pemphigus. We also acknowledge data management and analysis support from Bellsystem24 Inc and EPS Co Ltd, Tokyo, Japan.

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APPENDIX

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SPECIAL NOTICE REGARDING CASE REPORTS

The *Journal of the American Academy of Dermatology*, like other medical journals, receives many more case reports than we are able to publish. To accommodate our authors and to give our readers access to a more diverse collection of interesting cases, effective January 1, 2009 all case reports must be submitted in the more abbreviated case letter format. A full description of the case letter format can be found in the most recent written and online Instructions for Authors.

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Clinica Chimica Acta 410 (2009) 13–18

Contents lists available at ScienceDirect

Clinica Chimica Acta

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Development of ELISA for the specific determination of autoantibodies against envoplakin and periplakin in paraneoplastic pemphigus

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ARTICLE INFO

Article history:
Received 7 August 2009
Received in revised form 27 August 2009
Accepted 27 August 2009
Available online 6 September 2009

Keywords:
Desmosome
Diagnosis
Autoantibody
Immunofluorescence microscopy

ABSTRACT

Introduction: In paraneoplastic pemphigus (PNP), indirect immunofluorescence microscopy using rat bladder sections is widely used to search for circulating autoantibodies which are predominantly directed against envoplakin and periplakin. A sensitive and specific detection system for envoplakin/periplakin-specific autoantibodies is not yet available.

Methods: Overlapping fragments spanning envoplakin and periplakin, respectively, were analyzed for their ability to bind PNP-specific autoantibodies by immunoblotting and ELISA in sera from patients with PNP ($n=31$), pemphigus vulgaris ($n=30$), and bullous pemphigoid ($n=50$) as well as healthy volunteers ($n=140$). The results were compared with those obtained by immunoblotting of extract of cultured human keratinocytes.

Results: Immunoblot analysis revealed that most sera contained antibodies against the N-termini of both plakins as well as against the C-terminus of envoplakin. By ELISA, reactivities against envoplakin$_{1-481}$, envoplakin$_{1626-2033}$, and periplakin$_{1-324}$ were found in 25 (80.6%), 25 (80.6%), and 23 (74.2%) PNP sera, and in 1 (1.2%), 3 (3.7%), and 2 (2.5%) control sera, respectively.

Conclusions: The new ELISA based on an N-terminal fragment of envoplakin shows a high diagnostic accuracy to detect circulating autoantibodies in PNP. It is easy to be setup and standardized and can help to differentiate PNP patients from those with pemphigus vulgaris.

1. Introduction

Paraneoplastic pemphigus (PNP) is an autoimmune mucocutaneous blistering disease first described by Anhalt et al. [1]. The disease is always associated with a tumor, most commonly with non-Hodgkin's lymphoma, chronic lymphocytic leukemia, Castleman's disease, and thymoma [1–3]. In addition to autoantibodies directed against components of the desmosome, i.e. desmogleins 1 and 3, sera from patients with PNP contain IgG directed against non-stratified epithelia, in particular transitional epithelium of the bladder. These PNP-associated antibodies target five proteins of the desmosomal and hemidesmosomal plaque, including desmoplakins I/II (250 and 210 kDa), BP230 (230 kDa), envoplakin (210 kDa), and periplakin (190 kDa), and a yet unidentified 170 kDa protein [1,2,4–7]. All of the identified antigens are constituents of the cornified cell envelope [8,9] and belong to the plakin family of proteins characterized by a central rod domain linking a globular N-terminal spectrin-repeat domain to a globular C-terminal plectin-repeat domain (Fig. 1).

Earlier investigations by immunoblotting of recombinant glutathione-S-transferase (GST) fusion proteins revealed that epitopes on envoplakin and periplakin targeted by PNP autoantibodies are distributed over various regions of both proteins with the exception of the C-terminus of periplakin [10]. Subsequent studies focused on the C-terminal linker domain of envoplakin and showed that the majority of sera from patients with PNP contained antibodies directed against this region and that, at least in individual cases, neoplasms may produce them [11]. Based on these data, two immunoassays employing the linker domain—a bead-based assay based on a protein expressed in bacteria and an ELISA based on a protein expression in a human cell line—have been reported recently [12,13]. Use of these systems in routine diagnostic is hampered by the complex setup of the

Abbreviations: PNP, paraneoplastic pemphigus; PV, pemphigus vulgaris; PF, pemphigus foliaceus; BP, bullous pemphigoid; NC16A, extracellular part of the 16th non-collagenous domain of BP180; IIFM, indirect immunofluorescence microscopy; DIFM, direct immunofluorescence microscopy; OD, optical density; ROC, receiver-operating characteristics; AUC, area under the curve; RU, relative units; HRP, horse radish peroxidase; TMB, tetramethylbenzidine; IMAC, immobilized metal chelate affinity chromatography; SDS, sodium dodecylsulfate; PAGE, polyacrylamide gel electrophoresis.

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doi:10.1016/j.cca.2009.08.022

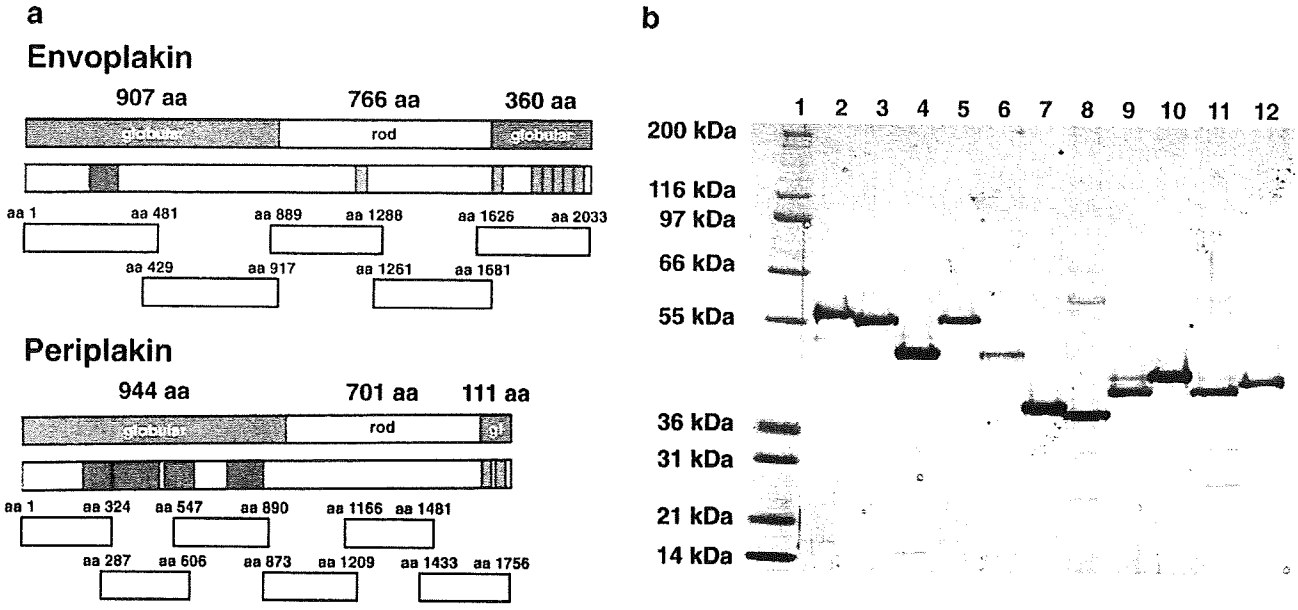

Fig. 1. Recombinant fragments of human envoplakin and periplakin generated for this study. (a) Schematic view on the globular (green) and rod (white) domain structures of envoplakin and periplakin and the fragmentation strategy used in this study. Spectrin repeats are depicted in red, plectin repeats in blue. (b) Recombinant fragments of envo- and periplakin were separated by SDS-PAGE and stained by Coomassie Blue: envoplakin$_{1-481}$ (lane 2, calculated mass: 55 kDa), envoplakin$_{429-917}$ (lane 3, calculated mass: 56 kDa), envoplakin$_{889-1288}$ (lane 4, calculated mass: 48 kDa), envoplakin$_{1261-1681}$ (lane 5, calculated mass: 52 kDa), envoplakin$_{1626-2033}$ (lane 6, calculated mass: 47 kDa), periplakin$_{1-324}$ (lane 7, calculated mass: 40 kDa), periplakin$_{287-606}$ (lane 8, calculated mass: 38 kDa), periplakin$_{547-890}$ (lane 9, calculated mass: 41 kDa), periplakin$_{873-1209}$ (lane 10, calculated mass: 42 kDa), periplakin$_{1166-1481}$ (lane 11, calculated mass: 39 kDa), and periplakin$_{1433-1756}$ (lane 12, calculated mass: 40 kDa). Migration positions of weight markers of 200, 116, 97, 66, 55, 36, 31, 21, and 14 kDa are shown in lane 1. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

bead assay that needs a sophisticated machinery in the first case and the necessity of an elaborate expression system in the other case.

In the present study, we re-investigated the epitope distribution on envoplakin and periplakin in a large number of PNP patients. Evenly truncated fragments with a small, less-immunogenic fusion partner that allowed purification by affinity chromatography were expressed in *E. coli*. These protein fragments were then used to form the solid phase in ELISA which allowed statistical analysis of the resulting quantitative data—in contrast to the formerly employed immunoblots—and the comparison of sensitivities and specificities of the different immunoassays. We demonstrate that an ELISA using a recombinant 56 kDa N-terminal fragment as the target antigen detects circulating autoantibodies in 80% of PNP sera at a specificity of ≥98%.

2. Materials and methods

2.1. Human sera

Serum samples were obtained retrospectively from patients with PNP in Germany ($n=5$), China ($n=8$), and Japan ($n=18$, sum: $n=31$). Sera from controls with pemphigus vulgaris (PV, $n=30$), and bullous pemphigoid (BP, $n=50$) before initiation of systemic treatment, as well as from healthy volunteers ($n=140$) were obtained in Germany.

PNP patients revealed at least 4 of the following 5 criteria: (i) severe and extensive erosive stomatitis, (ii) a neoplasm, (iii) suprabasal acantholysis and cleft with scattered necrotic keratinocytes by histopathological examination, (iv) an intercellular epidermal staining pattern of a perilesional biopsy by direct immunofluorescence microscopy (DIFM), (v) and serum IgG antibodies that bound intercellularly to the epithelium of monkey esophagus as well as on rat bladder epithelium by IIFM (EUROIMMUN, Lübeck, Germany).

Patients with PV had (i) oral erosions, (ii) IgG autoantibodies that showed an intercellular staining pattern by DIFM of a perilesional biopsy, and (iii) circulating IgG that intercellularly labeled the epithelium of monkey esophagus but were unreactive with rat bladder by IIFM (EUROIMMUN), and (iv) serum IgG antibodies against desmoglein 3 detected by ELISA performed according to the manufacturer's instructions (MBL, Nagoya, Japan).

Patients with BP had developed (i) tense blisters and erosions, (ii) no predominant mucosal lesions, (iii) IgG autoantibodies that bound along the dermal–epidermal junction by DIFM of a perilesional skin biopsy, and (iv) serum IgG antibodies to the extracellular part of the 16th non-collagenous domain (NC16A) of type XVII collagen/BP180 by ELISA performed according to the manufacturer's instructions (EUROIMMUN). Sera from healthy volunteers ($n=140$) were unreactive by IIFM on monkey esophagus as well as rat bladder.

All samples were collected between 2004 and 2007. The study was approved by the ethics committee of the Medical Faculties of the University of Lübeck (Institutional board projects 04-143, 06-090). In adherence to the Helsinki principles, we obtained written informed consent from all patients. All serological tests necessary for the characterization of the patients were performed at the Department of Dermatology of University of Lübeck. Clinical data was obtained in the center of appearance.

2.2. Keratinocyte extract

HaCaT keratinocytes were kindly provided by N.E. Fusenig (Heidelberg, Germany) and grown as described [14]. Confluent monolayers were extracted with NuPAGE sample buffer (Invitrogen, Karlsruhe, Germany) supplemented with 5 mM EDTA and 10 mM DTT. Extracts were pulse-sonicated on ice, centrifuged, and the supernatants aliquoted and stored at −70 °C until used.

2.3. Construction of recombinant vectors encoding for fragments of human envoplakin and periplakin

DNA sequences coding for five overlapping fragments of envoplakin (Acc No. U53786) and six fragments of periplakin (Acc No. AF001691), respectively, were integrated into the prokaryotic expression vector

pET24d-N [15] thereby adding N-terminal hexahistidine-tags to the recombinant proteins. Coding cDNA fragments were obtained by i) overlap extension PCR [16], ii) PCR-based gene synthesis, or iii) commercially (Imagenes, Berlin, Germany). Primers for polymerase chain reactions (PCR), designed to contain appropriate restriction sites, were synthesized by MWG Biotech (Ebersberg, Germany).

In brief, envoplakin$_{1-481}$ and envoplakin$_{429-917}$ cDNAs were assembled by overlap extension PCR from eleven and six overlapping exon spanning fragments, respectively, that had been generated either from genomic DNA of HEK293 or commercially available cDNA (Supplementary Tables 1 and 2). Periplakin$_{1-324}$ cDNA was generated by fusion of a synthetic DNA to a conventional cDNA amplification product based on commercially available cDNA (Supplementary Tables 1 and 2). Coding fragments for envoplakin$_{889-1288}$, envoplakin$_{1261-1681}$, envoplakin$_{1626-2033}$, periplakin$_{287-606}$, periplakin$_{547-890}$, periplakin$_{873-1209}$, periplakin$_{1166-1481}$, and periplakin$_{1433-1756}$ were amplified directly either from the 22nd exon or from commercially available cDNA (Supplementary Tables 1 and 2). PCR reactions and subsequent ligations with pET24d-N were carried out as described [15] with the modification that the PCR extension time was adjusted to 60 s extension per 1 kb amplification product size.

2.4. Expression and purification of recombinant envoplakin and periplakin fragments

The eleven fragments (Fig. 1a) were expressed in *E. coli* Rosetta (DE3) pLacI (Novagen, Darmstadt, Germany). For this purpose, a fresh bacterial colony was used to inoculate 10 ml LB medium supplemented with 50 µg/ml kanamycine and 34 µg/ml chloramphenicol. The culture was incubated at 37 °C for 12 h in a bacterial shaker at 130 rpm. The primary culture was used to inoculate 500 ml LB medium with appropriate antibiotics and incubated as described until the bacterial suspension reached an $OD_{600\ nm}$ of 0.5–0.6. Protein expression was induced with 1 mM isopropyl D-thyogalactopyranoside for 3 h. Bacteria were harvested by centrifugation at 1800×g for 30 min and sediments were resuspended in 10 ml of 10 mM Tris–Cl pH 8.0, 0.3 M NaCl, 1 M EDTA. To release the recombinant proteins, the cell suspension was sonicated 3 times 20 s (Branson Sonifier) on ice immediately after addition of Triton X-100 (Sigma) to an end concentration of 1% (w/v). Lysed bacteria were centrifuged again at 15,000×g for 20 min to sediment insoluble proteins which were subsequently solubilized in 10 ml 5 mM Tris–Cl pH 8.0, 0.3 M NaCl, 8 M urea, 5 mM imidazole. The respective recombinant protein was purified by immobilized metal chelate affinity chromatography (IMAC) using Ni-NTA sepharose (Qiagen) and AU buffer (50 mM sodium acetate pH 4.5, 8 M urea) as eluents. As a further step of purification, eluates were directly applied onto a cation exchange resin (SP-sepharose, GE Healthcare, Freiburg, Germany) equilibrated with AU buffer. After thorough washing with AU buffer, fragments were eluted with 50 mM sodium phosphate pH 7.4, 1 M NaCl, 8 M urea. Protein concentrations were determined by bicinchoninic acid assay (Sigma, Taufkirchen, Germany). The proteins, purified by IMAC and cation exchange chromatography, migrated at their calculated size when separated by SDS-PAGE (Fig. 1). A monoclonal antibody specific for hexahistidine (Merck Biosciences, Darmstadt, Germany) recognized the recombinant fragments of envo- and periplakin by immunoblotting. Identity, full length of the recombinant protein, and purity of the preparations were verified by MALDI-ToF analysis (IndyMed, Rostock, Germany).

2.5. Generation of rabbit sera against envoplakin and periplakin

Due to the absence of a commercially available periplakin-specific monoclonal antibody, envoplakin$_{889-1288}$ and periplakin$_{873-1209}$ (Fig. 1), respectively, were used to immunize rabbits to generate antigen-specific polyclonal sera with the internal standard protocol and adjuvant of Eurogentec (Seraing, Belgium). The reactivities and mono-specificities of anti-envoplakin$_{889-1288}$ and anti-periplakin$_{873-1209}$ rabbit sera were evaluated by IIFM on monkey esophagus and rat bladder (Fig. 2a–d) and by immunoblotting of keratinocyte extract (Fig. 2e and f) and recombinant fragments (data not shown).

2.6. ELISA by the use of envoplakin and periplakin fragments

Microtiter plates (Nunc, Langenselbold, Germany) were saturated with the periplakin or envoplakin fragments serially diluted in PBS (50 mM sodium phosphate pH 7.4, 150 mM sodium chloride) overnight at 4 °C, treated with washing buffer (0.05% (w/v) Tween-20 in PBS), and blocked with blocking buffer (0.1% (w/v) casein in PBS) for 2 h. Saturation of the plates was analyzed by incubation with a murine monoclonal anti-his-tag antibody diluted 1:2000 in sample buffer (0.05% (w/v) Tween-20, 1% (w/v) casein in PBS) for 30 min. After adding 200 µl of washing buffer 3 times, the bound antibody was detected by incubation with anti-mouse IgG HRP conjugate (Dianova, Hamburg, Germany), diluted 1:2000 in sample buffer, for 30 min, subsequent washing as described above, followed by addition of TMB substrate (EUROIMMUN) for 15 min. Reactivity of human sera was analyzed using the same procedure, except for a different dilution of the serum (1:200) and the use of an anti-human IgG HRP conjugate (EUROIMMUN). All steps were carried out at room temperature. The OD was read at 450 nm using an automated spectrophotometer (Tecan, Crailsheim, Germany).

2.7. Indirect immunofluorescence microscopy

IIFM was conducted using slides with primate esophagus and rat bladder according to the manufacturer's instructions (EUROIMMUN). Slides were evaluated by two independent persons. In case of discrepant results a third person was involved for a decisive evaluation.

2.8. Miscellaneous

All tests were performed at the Institute for Experimental Immunology, EUROIMMUN AG except otherwise stated. All antibody measurements were performed under code and blinded to clinical data by personnel with at least three years of training in 2008. All statistical analyses (ROC analysis, correlation analysis) were performed using the EUROStat statistical package (EUROIMMUN).

3. Results

3.1. Reactivity of patients' sera by immunoblotting with keratinocyte extract

Sera from PNP ($n=31$) and PV patients ($n=30$) and 30 healthy volunteers were analyzed by immunoblotting with cellular extract of cultured HaCaT cells. Migration positions of envoplakin and periplakin were determined by anti-envoplakin$_{889-1288}$ and anti-periplakin$_{873-1209}$ rabbit sera (Fig. 2). All PNP sera ($n=31$) displayed reactivity with either envoplakin ($n=30$) or periplakin ($n=29$). In the control group, one PV serum contained antibodies specific to both envoplakin and periplakin but was unreactive by IIFM on both rat and monkey bladder sections. In addition, in this patient, a tumor work-up, including chest X-ray, ultrasound of abdomen as well as cervical, axillary, and inguinal lymph nodes, and analysis of stool for occult blood was negative.

3.2. Preparation of recombinant proteins

DNA sequences coding for fragments of human envoplakin and periplakin were cloned into prokaryotic expression vectors and expressed in *E. coli*. By SDS-PAGE, proteins purified by IMAC and cation exchange chromatography, migrated at expected positions with the exception of envoplakin$_{1626-2033}$ (calculated mass 52 kDa) which

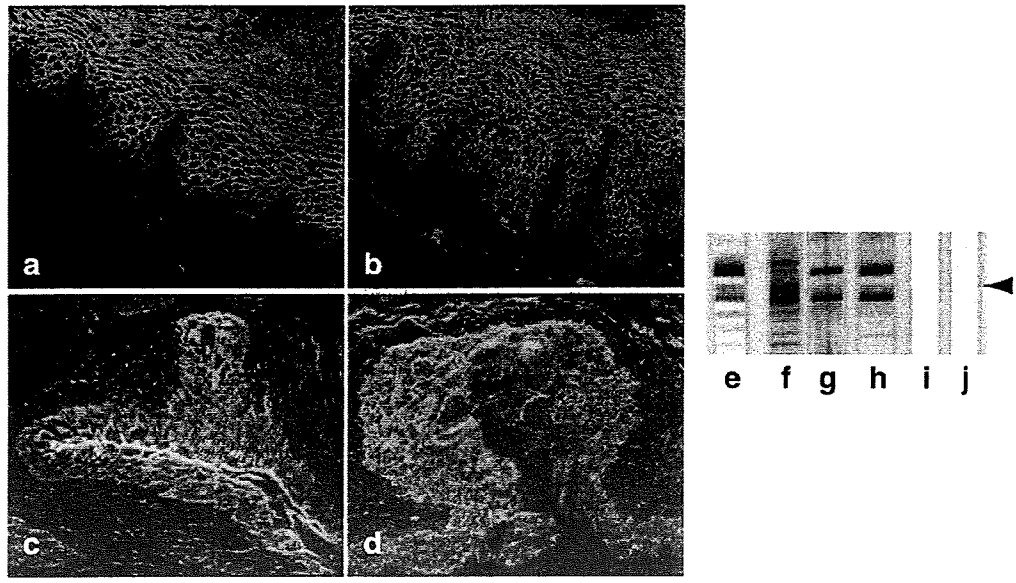

Fig. 2. Rabbit sera raised against envoplakin$_{889-1288}$ and periplakin$_{873-1209}$ stain monkey esophagus and rat urinary bladder by indirect immunofluorescence microscopy and proteins of 210 and 190 kDa by immunoblotting. Cryosections of monkey esophagus (a, b) or rat urinary bladder (c, d) were incubated with rabbit sera (diluted 1:80), generated by immunization against envoplakin$_{889-1288}$ (a, c) or periplakin$_{873-1209}$ (b, d), respectively. Membrane strips with electrophoretically separated keratinocyte extract were incubated with either rabbit sera (diluted 1:500), generated by immunization against envoplakin$_{889-1288}$ (e) or periplakin$_{873-1209}$ (f), or human sera (diluted 1:200) from patients with PNP (g, h) and from healthy blood donors (i, j). The arrowhead depicts the position of the 200 kDa marker protein.

showed a migration behaviour identical to envoplakin$_{429-917}$ (calculated mass 56 kDa, Fig. 1). MALDI-ToF in-source-decay analysis revealed the presence of correct N- and C-termini in all recombinant fragments thereby conforming the full length of the respective fragment after purification. Of the eleven fragments, only envoplakin$_{889-1288}$ and periplakin$_{873-1209}$ were recognized by the respective polyclonal rabbit sera raised against these fragments (data not shown).

3.3. Optimization of the envoplakin and periplakin ELISA

Microtiter plates were coated with recombinant proteins using saturating concentrations for each individual protein. Saturating concentrations were determined by measuring the reactivity of a monoclonal antibody specific for hexahistidine at different coating concentrations. The point of saturation for each polypeptide was defined as the minimal concentration leading to a maximal signal in the individual saturation curve. Saturating concentrations varied between 1 μg/ml (envoplakin$_{1-481}$) and 11 μg/ml (periplakin$_{1-324}$).

Coated microtiter plates were analyzed for the ability to immobilize PNP-associated antibodies in 46 sera (23 PNP, 23 healthy blood donors) and the resulting quantitative data were subjected to ROC analysis for the determination of AUC values for the individual polypeptides (Table 1). With the exception of antibodies against periplakin$_{287-606}$ and periplakin$_{547-890}$, the presence of autoantibodies displayed a significant relation to PNP. Based on the ROC data, cut-off values were defined at a specificity of 95.7%.

3.4. Analysis of ELISA performances

When evaluated at a specificity of 95.7%, the ELISA using fragments envoplakin$_{1-481}$, envoplakin$_{1626-2033}$, or periplakin$_{1-324}$, respectively, showed the highest sensitivities (Table 1). These 3 ELISA were subsequently used for the determination of autoantibody levels in a large cohort of patients and controls (31 PNP, 30 PV, 50 BP, and 140 healthy controls).

Of the 31 PNP sera, 25 showed antibodies against envoplakin$_{1-481}$ and envoplakin$_{1626-2033}$. Of these 25, 23 also contained antibodies against periplakin$_{1-324}$. A high correlation was seen between ELISA results obtained with envoplakin$_{1-481}$ or periplakin$_{1-324}$ as target antigens ($R = 0.9574$), whereas readings in both assays correlated weakly with reactivities obtained using the envoplakin$_{1626-2033}$ ELISA ($R = 0.6763$ and $R = 0.6922$). Envoplakin$_{1-481}$ in the sample buffer inhibited the reactivities of 5 representative sera in the anti-envoplakin$_{1-481}$ ELISA (inhibition: 85–89% at 10 μg/ml antigen) as well as in the anti-periplakin$_{1-324}$ ELISA (inhibition: 82–87% at 10 μg/ml antigen) in a concentration-dependent manner.

In the control group, antibodies to envoplakin$_{1-481}$ were detected in 1 PV serum (3.3%), none of the BP sera (0%) and 1 serum from a healthy volunteer (1.4%). The 2 positive sera only slightly exceeded the cut-off (Fig. 3). The ELISA using anti-envoplakin$_{1626-2033}$ also produced low scores in 1 PV serum (3.3%), 2 BP sera (4%) and 2 sera from healthy subjects (1.4%). Finally, anti-periplakin$_{1-324}$ antibodies were detected at considerable levels in 1 PV (3.3%), 1 BP (2%) and 8 sera from healthy subjects (5.7%). The PV serum containing anti-envoplakin$_{1-481}$ and anti-periplakin$_{1-324}$ reactivity also labeled envoplakin by immunoblotting of cellular extracts of cultured keratinocytes, but was not positive by ELISA using envoplakin$_{1626-2033}$.

Table 1
ROC analysis data based on reactivity of 23 sera from patients with PNP and 23 sera from healthy blood donors with the different recombinant fragments of envo- and periplakin.

	AUC	P	95% CI	Sensitivity [%] at 95.7% specificity	Sensitivity [%] at 100% specificity
Envoplakin$_{1-481}$	0.957	<0.0001	0.891–1.000	91.3	82.6
Envoplakin$_{429-917}$	0.919	<0.0001	0.842–0.995	69.6	52.2
Envoplakin$_{889-1288}$	0.878	<0.0001	0.782–0.974	65.2	52.2
Envoplakin$_{1261-1681}$	0.862	<0.0001	0.760–0.964	60.9	52.2
Envoplakin$_{1626-2033}$	0.949	<0.0001	0.889–1.000	82.6	69.6
Periplakin$_{1-324}$	0.917	<0.0001	0.837–0.997	82.6	47.8
Periplakin$_{287-606}$	0.549	0.2855	0.379–0.719	17.4	0.0
Periplakin$_{547-890}$	0.614	0.0868	0.450–0.779	26.1	21.7
Periplakin$_{873-1209}$	0.779	<0.0001	0.642–0.916	43.5	43.5
Periplakin$_{1166-1481}$	0.759	0.0001	0.619–0.899	47.8	43.5
Periplakin$_{1433-1756}$	0.726	0.0013	0.579–0.873	43.5	26.1

CI, confidence interval.

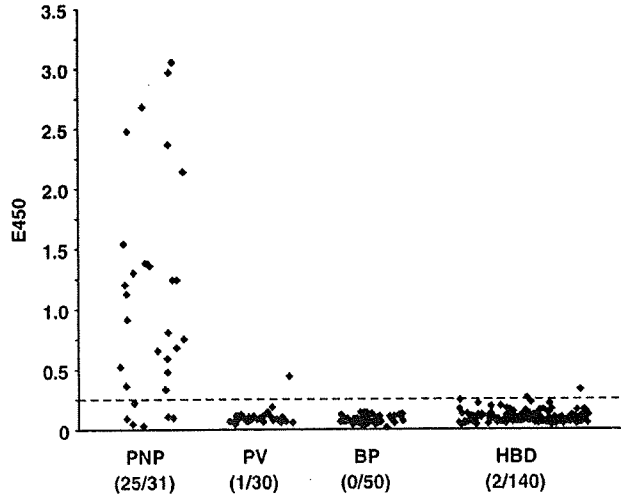

Fig. 3. The ELISA using envoplakin$_{1-481}$ shows a high sensitivity and specificity for the detection of autoantibodies in PNP sera. Sera from patients with paraneoplastic pemphigus (PNP) and control sera from patients with pemphigus vulgaris (PV), bullous pemphigoid (BP), and healthy blood donors (HBD) were incubated at a 100-fold dilution with immobilized envoplakin$_{1-481}$. Each serum was tested in triplicate and plotted points represent optical density at 450 nm. The cut-off of the assay is represented by a dotted line. Numbers in brackets show positive and total number of sera tested, respectively.

Parameters of ELISA performance were calculated on the basis of the 31 PNP and 80 control sera (PV, $n=30$; BP, $n=50$). Sensitivities and specificities of the ELISA systems were 80.6% and 98.8% (envoplakin$_{1-481}$), 80.6% and 97.5% (envoplakin$_{1626-2033}$), and 74.2% and 96.3% (periplakin$_{1-324}$), respectively. Diagnostic accuracies were 93.7% ($p<0.0001$), 92.8% ($p<0.0001$), and 90.1%, respectively ($p<0.0001$, Fisher's exact test) (Fig. 4).

4. Discussion

PNP and PV frequently share the clinical finding of a severe stomatitis and the presence of serum antibodies to desmoglein 3. Autoantibodies to envoplakin and periplakin are found in almost all PNP patients [6] and are rarely detected in non-PNP sera [17]. When skin blisters appear in PNP, both, patient and treating practitioner, are often unaware of an associated tumor. Therefore, the differentiation between PV and PNP is helpful to quickly initiate the search for the tumor, especially in the case of a malignant type, once PNP-specific antibodies are detected. We here report the development of a simple, specific and sensitive detection system for the detection of anti-periplakin and anti-envoplakin.

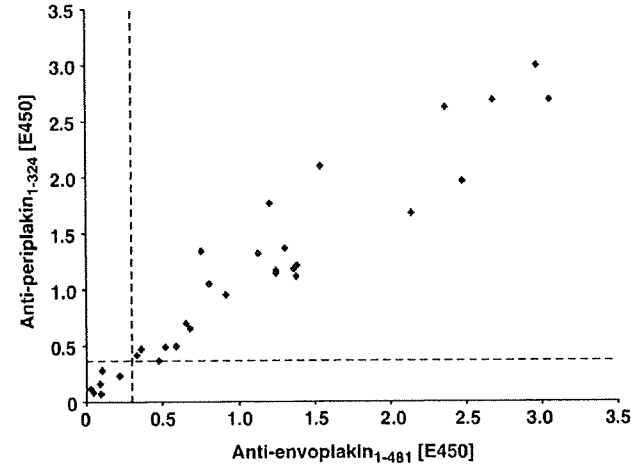

Fig. 4. Correlation of reactivities against envoplakin$_{1-481}$ and periplakin$_{1-324}$ indicates cross-reactive epitopes. Sera from patients with paraneoplastic pemphigus were incubated at a 100-fold dilution with immobilized envoplakin$_{1-481}$ and periplakin$_{1-324}$, respectively. Each serum was tested in triplicate and plotted points represent optical density at 450 nm. The cut-offs of the assays are represented by dotted lines. Serum reactivities against envoplakin$_{1-481}$ and periplakin$_{1-324}$ showed a high correlation factor, $R=0.9574$.

For several years, detection of circulating autoantibodies in PNP relied on immunoprecipitation of radiolabeled keratinocytes, immunoblotting of keratinocyte extracts, and, as a screening method, on IIFM on monkey or rat bladder [1–5]. In many laboratories, these tools are not available and they are difficult to standardize between different centers. Subsequently, antibodies to envoplakin and periplakin have been identified as serologic markers with nearly similar specificities for PNP [2,6].

Recombinant fragments of envoplakin and periplakin, fused to GST from *Schistosoma japonicum*, have also been employed in immunoblot analyses for detection of PNP autoantibodies [10]. Protocols employing GST fusion proteins bear the risk of false positive reactivity due to *Schistosoma* infection or, in the future, use of currently developed GST-based vaccines [18]. Therefore, the aim of this study was the establishment of an easy-to-use and easy-to-standardize routine ELISA for the sensitive and specific detection of autoantibodies to envoplakin and periplakin.

In a first set of experiments, 5 fragments of envoplakin and 6 of periplakin were generated by genetic engineering and expressed in *E. coli*. The evenly sized polypeptides included hexahistidine oligopeptide sequences to simplify purification via IMAC and, in contrast to GST, to minimize the size of the heterologous fusion partner. Due to the absence of specific antibodies for both proteins, envoplakin$_{889-1288}$ and periplakin$_{873-1209}$ were used to produce polyclonal rabbit antisera for the identification of envoplakin and periplakin in extracts of cultured keratinocytes by immunoblotting. This immunoblotting approach was then used for the initial characterization of the study sera: 30 of our 31 PNP sera, but only 1 serum of the non-PNP group contained antibodies against proteins with a migration behaviour identical to envo- and periplakin. Interestingly, all positive PNP sera contained antibodies against envoplakin, whereas reactivity with periplakin was less frequent. Possible explanations for this observation include different antibody titers against the 2 autoantigens in patients' sera or different amounts of these proteins in the extract of cultured keratinocytes.

In line with previous findings, a large number of PNP sera contained antibodies against the C-terminal linker region of envoplakin [10–12], though we found the target epitopes to be broadly distributed on both proteins. In contrast to previous studies, we saw a predominance of PNP-specific epitopes on the N-terminal portion of envoplakin (envoplakin$_{1-481}$). In fact, all PNP sera, reactive with at least one of the 11 recombinant envoplakin and periplakin fragments, contained antibodies against envoplakin$_{1-481}$. In addition, most periplakin-reactive PNP sera recognized epitopes within the N-terminus of periplakin (anti-periplakin$_{1-324}$). In contrast, partly confirming previous findings, there was much less reactivity with the remaining periplakin fragments, including its C-terminal linker domain [10,11]. The high degree of correlation between the quantitative results obtained in ELISA using envoplakin$_{1-481}$ and periplakin$_{1-324}$ as well as the inhibition of anti-periplakin$_{1-324}$ reactivity by the larger envoplakin$_{1-481}$ fragment indicates the presence of epitopes which are conserved between the N-termini of envoplakin and periplakin which contain a homologous spectrin repeat. Due to the importance of this region for the assembly of the cornified envelope [19], antibodies against the N-termini of both proteins might therefore have a pathogenetic potential. The cross-reactivity of autoantibodies is also in line with recent reports on cross-reactivity of a monoclonal anti-envoplakin antibody with periplakin [11,12].

In a further experiment, we determined levels of autoantibodies to envoplakin by ELISA in a large number of sera from PNP patients and controls using N-terminal envoplakin$_{1-481}$. In this cohort, 80.6% of PNP sera revealed autoantibodies to this fragment ELISA at a specificity of 98.8%. Antibodies in the remaining PNP sera, which were also positive by IIFM on urinary bladder epithelium, may be directed against other proteins of the plakin family, e.g. desmoplakin II, which exhibits an identical migration behaviour in SDS-PAGE, or against potential post-translational modifications present on envoplakin which are absent in the bacterially expressed recombinant fragments.

One PV serum containing antibodies against the N-terminal fragments of both envo- and periplakin as measured by ELISA was verified to contain anti-envoplakin antibodies by immunoblotting, while no immunoblot reactivity with periplakin was detected. However, it was negative by IIFM on rat bladder epithelium and a thorough work-up did not show an associated malignancy. One may speculate that antibodies to envoplakin are also present in a subset of PV patients; in fact, antibodies to desmoplakins and periplakin have previously been reported in sera of patients with PV or toxic epidermal necrolysis [17,20,21]. Alternatively, cross-reactivity of anti-desmoglein antibodies with envoplakin may have occurred in our patient as previously described for patients with pemphigus foliaceus [22].

Recently, a study including 16 PNP sera identified anti-envoplakin and periplakin antibodies in all sera by the use of an ELISA based on C-terminal fragments of both proteins, expressed in mammalian cells [13]. Mammalian expression is an elaborate and expensive technique which may limit the routine use of an ELISA based on this expression system. In addition, the high specificity of this study may be due to the highly homogenous patient population: all patients were from China and all but 2 patients had Castleman's disease as an associated tumor, a disorder that is rare in Europe and North America. Future work may directly address the question, if the ethnic background, or the type of the associated tumor, influences autoantibody reactivity against envoplakin and periplakin.

In summary, we describe a sensitive, specific, and easy to perform ELISA for the detection of autoantibodies to envoplakin in patients with PNP. Its application may further facilitate the diagnosis of PNP and the differentiation from PV in the great majority of patients.

Acknowledgments

We thank Britta Auweck, Antje Friedrich, Stephanie Niemann, Beatrice Schneider, Doreen Weigel, and Beatrice Ziegler for their excellent technical assistance. This work was in part supported by the Werner und Klara Kreitz-Stiftung, Lübeck to A.R. and D.Z. and by the Excellence Cluster Inflammation@Interfaces (DFG EXC306/1).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cca.2009.08.022.

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