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Short Communication

Autoimmunity to Desmocollin 3 in Pemphigus Vulgaris

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Pemphigus vulgaris is a blistering disease associated with autoantibodies to the desmosomal adhesion protein, desmoglein 3. Genetic deficiency of desmoglein 3 in mice mimics autoimmunity to desmoglein 3 in pemphigus vulgaris, with mucosal-dominant blistering in the suprabasal layer of the epidermis. Mice with an epidermal-specific deletion of desmocollin 3, the other major desmosomal cadherin isoform expressed in the basal epidermis, develop suprabasal blisters in skin that are histologically identical to those observed in pemphigus vulgaris, suggesting that desmocollin 3 might be a target of autoantibodies in some pemphigus vulgaris patients. We now demonstrate that desmocollin 3 is an autoantigen in pemphigus vulgaris, illustrated in a patient with mucosal-dominant blistering. Six of 38 pemphigus vulgaris and one of 85 normal serum samples immunoprecipitate desmocollin 3 ($P = 0.003$). Incubation of patient IgG with human keratinocytes causes loss of intercellular adhesion, and adsorption with recombinant desmocollin 3 specifically prevents this pathogenic effect. Additionally, anti-desmocollin 3 sera cause loss of keratinocyte cell surface desmocollin 3, but not desmoglein 3 by immunofluorescence, indicating distinct cellular pathogenic effects in anti-desmocollin and anti-desmoglein pemphigus, despite their identical clinical presentations. These data demonstrate that desmocollin 3 is a pathogenic autoantigen in pemphigus vulgaris and suggest that pemphigus vulgaris is a histological reaction pattern that may result from autoimmunity to desmoglein 3, desmocollin 3, or both desmosomal cadherins. *(Am J Pathol 2010, 177:2724–2730; DOI: 10.2353/ajpath.2010.100483)*

Pemphigus vulgaris (PV) is a potentially fatal autoimmune disease, classically associated with autoantibodies against the desmosomal cadherin, desmoglein (Dsg) 3. Seven desmosomal cadherins, desmogleins 1–4 and desmocollins (Dsc) 1–3, have been described.[1] Dsg3 and Dsc3 are the predominant isoforms expressed in the basal epidermis, which is the site of blister formation in PV. Dsg1, Dsg4, and Dsc1 are expressed in an inverted pattern, predominantly in the superficial epidermis with low to undetectable levels in the basal layers. Dsg2 and Dsc2 have weak to undetectable expression levels in the basal skin epidermis. Homophilic interactions between the extracellular domains of Dsg3 confer adhesion in cell aggregation assays[2]; both cell aggregation assays and functional data using adhesion-blocking peptides support the relevance of heterophilic interactions between desmogleins and desmocollins in desmosomal adhesion.[3–5] However, the relevant *in vivo* interactions of the desmosomal cadherins remain poorly understood.

The desmoglein compensation theory proposes that Dsg1 can compensate for the functional loss of Dsg3, and vice versa, regarding desmosomal cell adhesion, which in part explains the clinical and microscopic localization of blisters in PV.[6,7] Enzyme-linked immunosorbent assay (ELISA) studies have shown that patients with mucosal-dominant PV react mainly against Dsg3,[8–10] causing blisters in the basal layer of the mucous membranes where Dsg1 expression is minimal. In mucosal-dominant PV, Dsg1 in the basal layer of the skin epidermis is thought to compensate for the functional loss of Dsg3,

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thereby preventing cutaneous blistering. In support of this theory, PV patients who progress from mucosal dominant to mucocutaneous disease often develop anti-Dsg1 in addition to anti-Dsg3 autoantibodies.[11]

Genetic deficiency of Dsg3 in mice leads to suprabasal blistering of the mucosa and skin at sites of trauma, similar to findings in mucosal-dominant PV patients.[12] Subsequent studies have shown that pemphigus autoantibodies cause endocytosis of cell surface Dsg3, leading to its depletion from desmosomes[13–15] and supporting the hypothesis that autoantibody binding causes loss of desmoglein function. Recently, K14-Cre mediated deletion of Dsc3 in mouse epidermis was shown to cause suprabasal blisters in the skin that were histologically identical to those observed in PV patients.[16] These studies raised the question of whether Dsc3 might also be a target autoantigen in PV.

We have identified a mucosal PV patient who demonstrates pathogenic autoantibodies to Dsc3. Patient serum causes loss of cell surface Dsc3 but not Dsg3, in contrast to anti-Dsg3 PV serum, which causes internalization of Dsg3 but not Dsc3. Testing of additional sera confirms that Dsc3 is a significant autoantigen in PV.

Materials and Methods

All studies were performed under research protocols approved by the relevant institutional review boards.

Production and Purification of Recombinant Desmoglein and Desmocollin Proteins

Baculoviral vectors pET Dsg1E-3E and pET Dsc3E, encoding the extracellular domains of desmogleins 1–3 and desmocollins 2–3 expressing an E and 6× histidine tag,[9,17,18] were transfected into Sf9 cells using the BaculoGold expression system (BD Bioscience, San Diego, CA). Recombinant baculovirus was amplified for four passages in Sf9 cells, followed by infection of Hi5 cells for recombinant protein expression. Expression of proteins was confirmed by immunoblot with horseradish peroxidase-labeled anti-E tag antibody (Abcam, Cambridge, MA) and chemiluminescence detection (GE Health care, Uppsala, Sweden). In some experiments, recombinant proteins were purified by Talon cobalt-affinity chromatography (Clontech, Mountain View, CA) with imidazole elution, followed by buffer exchange into PBS containing 1 mmol/L $CaCl_2$.

Cell Culture

Primary human keratinocytes were isolated from neonatal foreskin by the Penn Skin Disease Research Core facility. Cells from passages 4 to 5 were cultured in defined keratinocyte serum free media (DK-SFM) (Invitrogen, Carlsbad, CA) supplemented with penicillin/streptomycin. Hi5 and Sf9 insect cells were cultured at 27°C in Express Five SFM or Sf-900 medium (Gibco, Carlsbad, CA), respectively, supplemented with Antibiotic-Antimycotic (Gibco) and L-glutamine.

Human Sera and Antibodies

The index patient developed mucosal erosions at age 49. Her past medical and family histories were unremarkable, and age-appropriate screening has shown no evidence of malignancy. Previously, her disease was successfully controlled with 50 mg azathioprine twice daily (1.6 mg/kg/day) and 60 mg prednisone daily (1 mg/kg/day). She also had a history of response to intramuscular gold and intravenous immunoglobulin in the past. The patient presented to clinic with a disease flare off-therapy. She was on no medications at the time of serum collection and responded well to systemic therapy with prednisone; she currently remains in clinical remission on 5 mg prednisone daily.

PV (n = 38) and normal (n = 78) sera were obtained from the University of Pennsylvania clinical laboratory. The diagnosis of PV was confirmed by clinical presentation, suprabasal blistering on histology, and positive direct and/or indirect immunofluorescence testing. Normal sera were derived from citrated human plasma samples by incubating with 120 mmol/L $CaCl_2$ for 2 hours at 37°C, followed by overnight incubation at 4°C and centrifugation to remove the resulting clot. IgG was purified from human serum samples using Melon Gel IgG spin purification systems (Pierce, Rockford, IL).

Additional normal (n = 7) and cell surface immunofluorescence-positive (n = 18) sera were obtained from the University of Utah clinical immunodermatology laboratory; of the latter, seven were Dsg3 ELISA-negative and 11 were Dsg3 ELISA-positive.

Antibodies used in this study include: mouse monoclonal anti-Dsg3 (5G11, Invitrogen), mouse monoclonal anti-Dsc3 (U114, Meridian Life Science Inc., Saco, ME), horseradish peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Lab, Inc., West Grove, PA); horseradish peroxidase-conjugated goat anti-E (Abcam); Alexa 488 or 594-conjugated goat anti-human IgG, and Alexa 488 or 594-conjugated donkey anti-mouse IgG (Molecular Probes, Carlsbad, CA).

Immunofluorescence Studies

Frozen sections (8 to 10 μm) of normal human skin were blocked in PBS containing 2% bovine serum albumin, 1 mmol/L $CaCl_2$, followed by incubation with primary antibody or human sera. Alexa 488- or 594-conjugated secondary antibodies were used to visualize binding of primary antibodies. For immuno-adsorption assays, human sera were diluted into 100 μl desmocollin or desmoglein baculoviral supernatant (containing approximately 500 ng recombinant protein) before incubation with human skin sections.

For immunofluorescence localization experiments, primary human keratinocytes were treated with antibodies in DK-SFM containing 1.2 mmol/L calcium for 4 hours at 37°C, then fixed for immunofluorescence as previously described.[15] Nuclear staining was performed with 4,6-diamidino-2-phenylindole (Sigma, St. Louis, MO; or Molecular Probes, Invitrogen). Immunofluorescence images

were acquired using a Hamamatsu Orca ER camera on an Olympus BX61 microscope with Slidebook 4.2 software. Nearest neighbor deconvolution was performed on the acquired images.

Immunoprecipitation

For recombinant proteins, 10 μl human sera were incubated with 100 μl baculoviral supernatant and 20 μl protein A or G beads (Invitrogen) overnight at 4°C. Primary keratinocyte lysates were obtained as previously described.[15] Triton X-100 soluble lysate (200 μl) was precleared with protein G beads, then incubated with 10 μl normal human or patient serum, or 5 μl Dsc3 mAb (U114) overnight at 4°C, followed by incubation with 20 μl protein G beads. Bound proteins were eluted with Laemmli sample buffer (BioRad, Hercules, CA) containing β-mercaptoethanol for 5 minutes at 100°C, separated by SDS-polyacrylamide gel electrophoresis, and detected by immunoblot using anti-Dsc3 (U114) or anti-E tag antibodies.

ELISA

Sera were tested for IgG Dsg3, Dsg1, BP180, and BP230 antibody reactivity by ELISA (MBL International, Woburn, MA) according to manufacturer's protocols, using a cut-off value of 20.0 and 9.0 for the desmoglein and BP180/230 ELISA, respectively. Anti-Dsg3 IgA was tested using horseradish peroxidase-anti-IgA secondary antibody (Dako, Carpinteria, CA).

Keratinocyte Dissociation Assay

Patient and normal human serum IgG (175 to 200 μg/ml) were evaluated for pathogenicity using a primary human keratinocyte dissociation assay as previously described.[19,20] Recombinant exfoliative toxin A produced in *Staphylococcus aureus* [1 μg/ml, generously provided by John Stanley[21]] was added to all human IgG dissociation experiments to inactivate Dsg1. For adsorption studies, patient IgG (100 μg) was pre-incubated with 25 μg purified recombinant Dsc3 or Dsg3 for 4 hours at 4°C. P1 pathogenic PV mAb (25 μg/ml) was used as a positive control.[15,20]

Results

A 55-year-old woman presented with gingival erosions (Figure 1A). The diagnosis of PV was confirmed by a maxillary gingival biopsy, demonstrating suprabasal acantholysis (Figure 1B) and indirect immunofluorescence on monkey esophagus indicating a high titer (1:2048) of circulating cell surface autoantibodies in the patient's serum. Unexpectedly, clinical testing for Dsg3 autoantibodies by ELISA was repeatedly negative. Additional ELISA testing indicated that the patient's serum was also negative for IgG Dsg1, BP180, and BP230 autoantibodies, as well as IgA Dsg3 autoantibodies (data not shown).

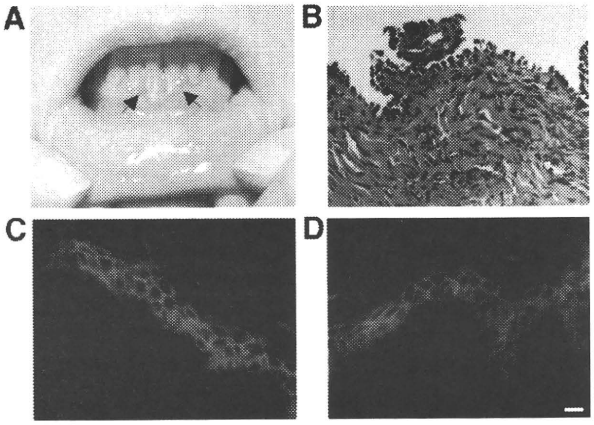

Figure 1. Clinical, histological, and immunochemical findings in a case of anti-desmocollin 3 (Dsc3) pemphigus. **A:** Characteristic findings of desquamative gingivitis (**arrows**) in a patient with mucosal pemphigus vulgaris (PV). **B:** H&E staining (×40 magnification) of a gingival biopsy, demonstrating suprabasal acantholysis. **C:** Indirect immunofluorescence (IIF) of patient serum on normal human skin, demonstrating IgG cell surface autoantibodies. **D:** Anti-Dsc3 mAb immunofluorescence on normal human skin, indicating a similar staining pattern compared to patient serum. Scale bar = 20 μm.

We hypothesized that the patient may have autoantibodies to Dsc3, the other major desmosomal cadherin isoform expressed in the basal epidermis. Indirect immunofluorescence of normal human skin using patient serum and a Dsc3 monoclonal antibody indicated a similar cell surface staining pattern, confirming the diagnosis of PV (Figure 1, C and D). To evaluate whether patient serum antibodies specifically bind Dsc3, we tested patient serum, normal human serum, and a known anti-Dsg3 PV serum for the ability to immunoprecipitate the recombinant extracellular domains of Dsc3 and Dsgs 1, 2, and 3 produced in baculovirus. Patient serum immunoprecipitated the recombinant extracellular domain of human Dsc3, but not Dsgs 1–3 (Figure 2A), while anti-Dsg3 pemphigus serum bound Dsg3 only, and normal serum did not react with any of the desmosomal cadherins. Additionally, patient serum did not bind Dsc1 or Dsc2 (data not shown). Patient serum, but not normal serum, also immunoprecipitated Dsc3 endogenously expressed in human keratinocytes (Figure 2B). Recombinant extracellular Dsc3 but not Dsg3 adsorbed all cell surface binding from the patient serum by immunofluorescence (Figure 2C), suggesting that Dsc3 is the primary target of serum cell surface autoantibodies.

To determine the prevalence of Dsc3 autoantibodies in PV, we tested 85 normal and 37 additional PV sera by immunoprecipitation using the recombinant extracellular domain of Dsc3. Six of 38 PV (15.8%) and one of 85 normal sera (1.2%) immunoprecipitated Dsc3, indicating a significant association of Dsc3 autoantibodies with PV (Table 1, $P = 0.003$ by Fisher's exact test). Three of 38 PV sera (7.9%) were positive by indirect immunofluorescence but negative for Dsg1 and Dsg3 autoantibodies by ELISA; of these samples, one (33.3%, the index patient) was positive for Dsc3 autoantibodies. To further examine immunofluorescence-positive, Dsg-negative sera, we obtained 18 additional cell surface immunofluorescence-positive sera

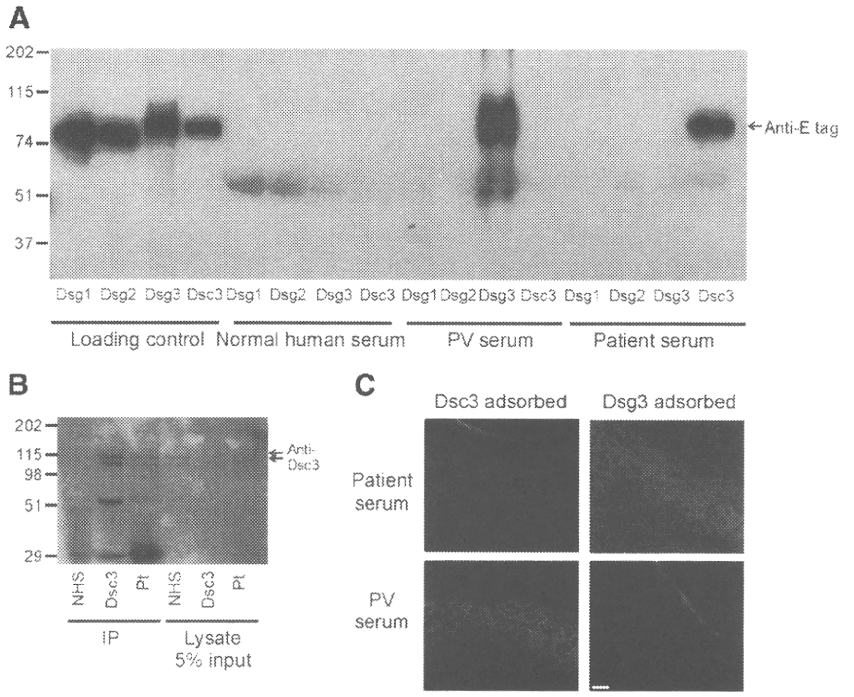

Figure 2. A: Patient serum immunoprecipitates recombinant Dsc3. Recombinant extracellular domains of desmogleins (Dsg) 1–3 and Dsc3 bearing an E tag were produced in a baculovirus expression system. Culture supernatants containing recombinant proteins were immunoprecipitated with normal human serum, anti-Dsg3 PV serum, or patient serum followed by immunoblotting using an anti-E tag detection antibody. PV serum bound Dsg3, while patient serum immunoprecipitated Dsc3, and normal serum did not react with any recombinant proteins. **B:** Patient serum recognizes Dsc3 endogenously expressed in human keratinocytes. Primary human keratinocyte lysates (200 ml) were immunoprecipitated using normal human serum (NHS), Dsc3 mAb, or the patient's serum, followed by immunoblotting with Dsc3 mAb. Endogenously expressed Dsc3 is detected as a doublet (corresponding to the "a" and "b" splice isoforms). Lower bands in lane 2 correspond to murine mAb heavy and light chains. Five percent input (10 ml) for each immunoprecipitation is shown in the right three lanes. **C:** Dsc3 is the primary target of cell surface autoantibodies. Patient serum or anti-Dsg3 PV serum was adsorbed with supernatants containing either Dsc3 or Dsg3 before immunostaining of human skin cryosections. Alexa-594 anti-human IgG secondary antibody was used to detect binding of serum autoantibodies to skin sections. Scale bar = 20 μm.

from the University of Utah clinical immunodermatology laboratory (seven Dsg3 negative and 11 Dsg3 positive by ELISA). Two of seven Dsg3-negative sera (28.6%) and three of 11 Dsg3-positive sera (27.2%) immunoprecipitated Dsc3. Of the 11 total Dsc3-reactive sera identified, eight reacted with both Dsc3 and Dsg3, while three reacted with Dsc3 only. A total of 10 Dsg3-negative sera were identified; three of these (30%) were positive for Dsc3. A summary of the serology and clinical phenotype of the six anti-desmocollin 3 PV patients identified at our clinical center appears in Table 2.

Anti-Dsc3 PV IgG caused the loss of cell adhesion in a primary human keratinocyte cell dissociation assay (Figure 3A). Adsorption of anti-Dsc3 PV IgG with the recombinant extracellular domain of Dsc3, but not Dsg3, prevented this pathogenic effect. Pathogenic anti-Dsg3 PV IgG and mAbs have previously been shown in human keratinocytes to cause internalization of Dsg3 into early endosomes, which are subsequently targeted to lysosomes.[13–15] We sought to determine whether patient autoantibodies have similar pathogenic effects on Dsc3. Incubation of the index patient serum with human keratinocytes caused loss of cell surface Dsc3 but not Dsg3 by immunofluorescence, the opposite of what is observed with anti-Dsg3 PV serum (Figure 3B). A second anti-Dsc3 patient serum showed similar results, with loss of cell surface Dsc3 but not Dsg3.

Table 1. Prevalence of Dsc3 Autoantibodies in Pemphigus Vulgaris and Normal Human Sera

	Desmocollin 3 positive		Desmocollin 3 negative		
	Dsg3−	Dsg3+	Dsg3+	Dsg3−	Total
Pemphigus vulgaris					
Penn	1*	5	30	2	38
Utah	2	3	8	5	18
Total	3	8	38	7	56
Normal	1		84		85

*Index patient.

Discussion

Pemphigus vulgaris is a disease of desmosomal cell adhesion, classically associated with autoantibodies against Dsg3. We present a case of anti-Dsc3 PV, illustrated in a patient with typical mucosal disease. Previously, atypical or paraneoplastic pemphigus patients with human Dsc3 immunoreactivity have been described,[17,22–24] although autoantibodies in these patients reacted with multiple skin antigens, and pathogenicity against Dsc3 was not demonstrated, raising the possibility that the Dsc3 autoantibodies may not have caused disease, but instead arose secondarily as a result of epidermal damage. Figure 2A and unpublished data in-

Table 2. Correlation of Clinical Phenotype and Serology in Anti-Dsc3 PV

Patient	Dsc3	Dsg3	Dsg1	Clinical phenotype
1*	+			Mucosal
2	+	+		Mucocutaneous
3	+	+		Mucosal
4	+	+	+	Mucocutaneous
5	+	+	+	Mucocutaneous
6	+	+	+	Mucocutaneous

*Index patient.

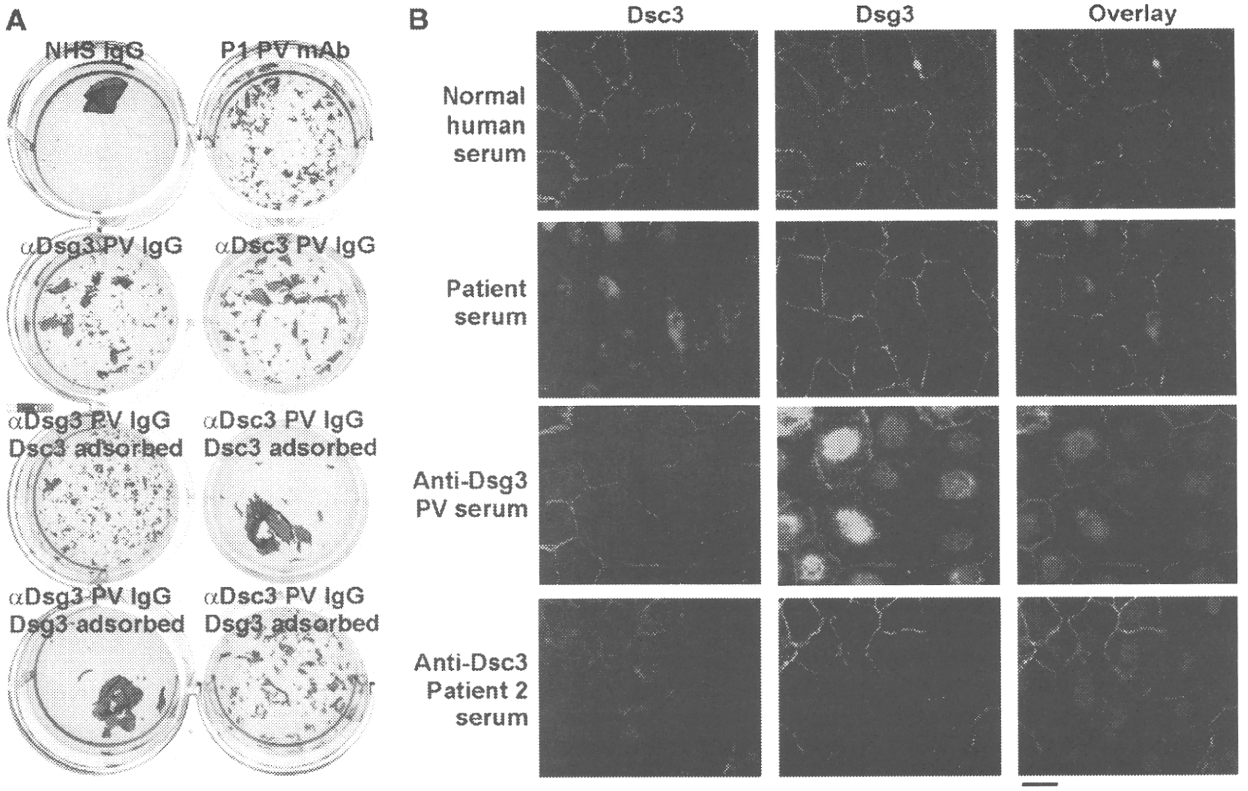

Figure 3. **A:** Anti-desmocollin 3 IgG causes loss of cell adhesion. Primary keratinocytes were incubated with anti-Dsg3 PV IgG or anti-Dsc3 PV IgG that had been pre-adsorbed with recombinant Dsg3 or Dsc3. Dsc3, but not Dsg3, adsorbed pathogenic activity from anti-Dsc3 PV IgG. NHS = normal human serum, P1 = positive control PV mAb. **B:** Distinct cellular phenotypes for anti-Dsc3 and anti-Dsg3 pemphigus. Primary human keratinocytes were treated with normal human serum, patient serum, anti-Dsg3 PV serum, or a second anti-Dsc3 patient serum for 4 hours during calcium-induced desmosome assembly. Cells were fixed and stained with a primary antibody specific to Dsc3 or Dsg3, followed by a secondary antibody conjugated with Alexa-594 (red) or Alexa-488 (green). Cell nuclei were stained with 4,6-diamidino-2-phenylindole. Anti-desmocollin pemphigus sera both demonstrate loss of cell surface Dsc3 but not Dsg3, while anti-desmoglein pemphigus serum causes loss of cell surface Dsg3 but not Dsc3. Scale bar = 10 μm.

dicate that our PV patient has autoantibodies only to Dsc3, and not Dsgs 1–3 or Dscs 1–2, which include all known desmosomal cadherin isoforms expressed in the basal epidermis. Affinity adsorption experiments (Figure 2C) further support that Dsc3 is the only keratinocyte cell surface antigen targeted by serum autoantibodies in this patient. Finally, pathogenicity studies indicate that patient IgG causes the loss of human keratinocyte cell adhesion through loss of cell surface Dsc3 but not Dsg3 (Figure 3). The effects on Dsc3, shown for two different anti-Dsc3 sera, precisely parallel the effects of anti-Dsg3 PV IgG and mAbs in regard to Dsg3 internalization, which has been shown to correlate with pathogenic activity.[13–15] Taken together, these data suggest distinct pathogenic mechanisms for anti-Dsg3 and anti-Dsc3 pemphigus, leading to the same histological presentation of suprabasal blistering.

To date, no human genetic blistering diseases have been documented in association with either Dsg3 or Dsc3. Germline deletion of Dsc3 in mice leads to preimplantation lethality before desmosomes are formed,[25] suggesting that Dsc3 deficiency may be incompatible with normal embryonic development, independent of its desmosomal adhesive function. Recently, a mutation in one family with hereditary hypotrichosis was associated with a nonsense mutation in Dsc3, predicted to occur at the junction of the transmembrane and cytoplasmic domains.[26] However, there was no histological evidence of skin blistering, and it is unknown whether truncated Dsc3 protein is expressed in these patients.[27] The finding of anti-Dsc3 autoantibodies in PV patients provides *in vivo* evidence from humans on the critical role of Dsc3 in epithelial cell adhesion.

Compelling evidence in mice also indicates that epidermal integrity requires both Dsg3 and Dsc3. Genetic loss of Dsg3 in mice causes suprabasal blisters similar to those in anti-Dsg3 PV patients, who demonstrate mucosal dominant disease.[12] Our anti-Dsc3 PV patient, like anti-Dsg3 PV patients, demonstrated mucosal but not skin disease. It is possible that other desmosomal cadherin isoforms such as Dsg1 and Dsg3 may compensate for the functional loss of Dsc3-mediated adhesion in skin, a hypothesis that has been experimentally validated for human Dsgs 1 and 3.[6,28]

Of the six anti-Dsc3 PV patients identified at our center, one (the index patient) demonstrated Dsc3 autoantibodies only, two had both Dsc3 and Dsg3 autoantibodies, and three had Dsc3, Dsg3, and Dsg1 autoantibodies (Table 2). All three patients with Dsc3, Dsg3, and Dsg1 autoantibodies had mucocutaneous disease. The index patient with Dsc3 autoantibodies demonstrated mucosal disease. Of the two patients with both Dsc3 and Dsg3

autoantibodies, one had mucocutaneous PV, and the other had mucosal only disease, although it is unknown whether the anti-Dsc3 autoantibodies were pathogenic in these patients. Extending the desmoglein compensation theory to include the desmocollins would predict that patients with Dsg3 and Dsc3 autoantibodies would be more likely to have skin blisters, since Dsg1-mediated adhesion alone may be insufficient for the maintenance of skin integrity in the basal epidermis. Future studies will be necessary to elucidate the clinical-serological correlation in anti-desmocollin and anti-desmoglein PV.

Targeted deletion of Dsc3 in mouse epidermis results in skin but not mucosal blistering,[16] which is distinct from the phenotype of our anti-Dsc3 PV patient. There could be multiple reasons for the discrepancy between the human autoimmune disease and mouse gene deletion model. Previous studies have shown that K14 transgenes may not be well expressed in oral mucosa.[29] Deletion of Dsc3 was only verified in mouse skin keratinocytes, raising the possibility that mucous membrane lesions were not observed in the K14-Cre;Dsc3 fl/fl mouse model due to retained Dsc3 expression in the oral mucosa. The discrepancy may also reflect variations in desmosomal cadherin isoform expression between humans and mice. Additionally, autoimmunity to Dsc3 has different mechanisms of pathogenicity as compared to genetic deficiency of Dsc3. In the autoimmune disease, pathogenic autoantibodies cause internalization of newly synthesized Dsg3, leading to Dsg3-depleted desmosomes that lose intercellular adhesion.[13–15,30] Genetic loss of Dsg3 would thus be predicted to have a more severe phenotype than autoimmunity to Dsg3, since skin keratinocytes would have no cell surface Dsg3 compared to a reduction in the steady-state level of cell surface Dsg3. Consistent with this finding, Dsg3-deficient mice demonstrate both mucosal erosions and skin erosions at sites of trauma, whereas passive transfer of pathogenic anti-Dsg3 autoantibodies does not cause skin blisters even after mechanical shear stress,[6,20] and anti-Dsg3 PV patients typically demonstrate mucosal disease only.[10,31]

In testing other normal (n = 85) and PV (n = 38) sera, we found a significant association of Dsc3 autoantibodies with PV (Table 1). To our knowledge this is the first systematic association of Dsc3 with human genetic or acquired blistering disease. Previous studies have not identified significant Dsc3 autoreactivity in the sera of PV patients. However, many of these studies used ELISA, which has been shown to be suboptimal for detecting autoreactive antibodies to desmocollin 1 in cases of IgA pemphigus,[32] potentially due to destruction or masking of epitopes. We have similarly found that autoreactive Dsc3 epitopes are not significantly detected by ELISA testing of pemphigus sera, in part due to the sensitivity of these epitopes to freezing and thawing (data not shown).

Interestingly, three of 38 PV sera (7.9%) in our initial screens were negative for Dsg3 autoantibodies by ELISA. This concurs with prior sensitivity data indicating that 85.2–97.5% of PV patients demonstrate serum Dsg3 autoantibodies by ELISA, depending on the cutoff value used for the assay.[9,33] We subsequently obtained seven additional Dsg3-negative, indirect immunofluorescence-positive sera from a clinical immunodermatology laboratory. Of the total combined 10 Dsg3-negative sera, three (30%) immunoprecipitated Dsc3. It is possible that the seven remaining Dsc and Dsg-negative sera may have an undetectably low titer of Dsg3 or Dsc3-reactive autoantibodies, as many of these samples had lower immunofluorescence titers. However, these findings also suggest that autoantigens other than Dsc3 and Dsg3 may exist in PV, as has been proposed.[34–36]

In summary, our findings suggest that PV is a histological reaction pattern that may result from autoimmunity to Dsg3, Dsc3, or both desmosomal cadherin isoforms, which may explain the site of blister formation in the basal epidermis where the expression patterns of these desmosomal cadherins overlap. Desmosomal cadherins are thought to engage primarily in heterophilic interactions between desmogleins and desmocollins, with weaker homophilic binding.[2,3,37] Recently, this theory has been challenged by the finding that Dsc3 and Dsg3 do not interact in a cell-free system.[38] The finding of similar clinical and histological phenotypes in anti-Dsc3 and anti-Dsg3 pemphigus supports a role for the biological interaction of these two desmosomal cadherins in the basal layers of the epidermis. Further studies are necessary to determine the relevant *in vivo* interactions of the human desmosomal cadherins.

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IgG Autoantibodies Against Desmocollin 3 in Pemphigus Sera Induce Loss of Keratinocyte Adhesion

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Pemphigus is considered an autoimmune bullous skin disorder associated with IgG against the desmosomal components, desmoglein 3 (Dsg3) and desmoglein 1 (Dsg1). This concept is supported by the *in vitro* and *in vivo* pathogenicity of anti-Dsg3/Dsg1 IgG and the mucosal blistering phenotype of mice with a genetic deficiency of Dsg3. Mice deficient for another desmosomal adhesion molecule, desmocollin 3 (Dsc3), show a similar pemphigus phenotype, and we investigated the pathogenicity of Dsc3-reactive IgG autoantibodies that were identified previously in a subset of patients with atypical pemphigus. We here demonstrate that IgG against Dsc3 causes loss of adhesion of epidermal keratinocytes. Specifically, IgG against Dsc3 was purified from Dsc3-reactive pemphigus sera by affinity column chromatography using recombinant human Dsc3. Affinity purified IgG was functionally active and did not only react with recombinant Dsc3 but also with epidermis and cultured human keratinocytes. Moreover, Dsc3-reactive IgG induced loss of adhesion of epidermal keratinocytes in a dispase-based keratinocyte dissociation assay that was reversed on pre-adsorption with human Dsc3 but not Dsg3. These findings demonstrate that IgG autoantibodies against an additional component of the desmosomes, Dsc3, induce loss of keratinocyte adhesion and thus may contribute to blister formation in pemphigus. ***(Am J Pathol 2011, 0: xxx; DOI: 10.1016/j.ajpath.2010.10.016)***

Pemphigus is a severe autoimmune bullous disorder of mucous membranes and skin which has been associated with autoantibodies (autoab) of the IgG class against two desmosomal components of epidermal keratinocytes, desmoglein 1 (Dsg 1) and desmoglein 3 (Dsg3).[1,2] Distinct clinical pemphigus variants, such as pemphigus vulgaris (PV) and pemphigus foliaceus (PF) have been linked to the presence of IgG reactive with their specific target antigens, Dsg3 and Dsg1, respectively.[3] Moreover, the clinical phenotype of these pemphigus variants can be largely explained by the tissue expression pattern of their autoantigens.[4] PV is characterized by suprabasal loss of adhesion of the mucous membranes, the site where Dsg3 is predominantly expressed.[5] In contrast, PF is recognized by superficial blisters of the skin but not of the mucosa because Dsg1 is preferentially produced by the subcorneal epidermis of stratified epithelia such as the skin.

In addition to the presence of four desmosomal Dsg isoforms, ie, Dsg1-4, epidermal desmosomes contain three different isoforms of another group of cadherins, namely desmocollin 1 (Dsc 1), desmocollin 2 (Dsc 2), and desmocollin 3 (Dsc 3).[6,7] Dsc1 has been previously identified as the target antigen of IgA autoab in the subcorneal pustular dermatosis type of IgA pemphigus.[8] Moreover, IgG autoab against Dsc1, Dsc2, or Dsc3 were detected in the sera of patients with paraneoplastic pemphigus and, occasionally, in patients with atypical pemphigus.[9–12] However, the pathogenic relevance of Dsc-specific autoab in these disorders remains unclear.

The concept that the pathogenesis of pemphigus is exclusively linked to IgG autoab against Dsg1 and Dsg3 has been challenged by the recent observation that mice with a genetic deficiency of Dsc3 show a pronounced blistering phenotype with suprabasal loss of epidermal adhesion that is highly reminiscent of PV.[13] It has been reported that Dsc3, as Dsg3, is preferentially expressed in the basal and suprabasal layers of human epidermis the site where loss of adhesion occurs in PV.[6,7,13] Dsg1 and Dsg3 not only interact in desmosomes via homophilic transinteraction, but presumably also interact by heterophilic binding with Dsc isoforms such as Dsc1 and Dsc3. However, an heterophilic transinteraction between Dsg3 and Dsc3 is unlikely based on recent *in vitro* findings.[14]

In the present study, affinity-purified, Dsc3-reactive IgG fractions from pemphigus sera induced loss of epidermal keratinocyte adhesion in a similar manner as pathogenic anti-Dsg3 IgG autoab. Thus, Dsc3 can be

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Table. Autoantibody Profile and Clinical Characteristics of the Studied Pemphigus Patients

Patient	Autoab isotype	Diagnosis	Clinical characteristics	Desmosomal target antigens*				
				Dsc1	Dsc2	Dsc3	Dsg1	Dsg3
1	IgG	Pemphigus vegetans	Exophytic skin erosions and oral erosions	–	–	+	–	–
2	IgG	Pemphigus vegetans	Hypertrophic verrucous plaques with pustules and erosions on foot, groin and scalp	–	–	+	+	–
3	IgG	Pemphigus herpetiformis	Multiple pustules, erythematous herpetiform erosions, oral erosions	–	–	+	–	–
4	IgG	Pemphigus herpetiformis	Erythematous cutaneous erosions	–	–	+	–	–

Autoab, autoantibody; Dsc, desmocollin; Dsg, desmoglein.
*Serum IgG reactivity as determined by immunoblot analysis using recombinant Dsg and Dsc proteins (see Figure 2).

considered as a relevant autoantigen of pemphigus and Dsc3-reactive IgG autoab may contribute to the blistering phenotype of pemphigus.

Materials and Methods

Patients

Sera of four patients with atypical pemphigus (pemphigus vegetans and pemphigus herpetiformis) were investigated. Assessment of the clinical conditions revealed exophytic skin erosions of the fingers and oral erosions (patient 1), hypertrophic vegetative (verrucous) plaques with pustules and erosions on the foot, groin and scalp (patient 2), widespread erythema, pustules and erosions of the oral mucosa (patient 3) and multiple erythematous erosions on trunk and extremities (patient 4). The clinical diagnosis of pemphigus was confirmed by: (a) histopathological evidence of intraepidermal loss of adhesion; (b) IgG deposits on the surface of epidermal keratinocytes of perilesional skin by direct immunofluorescence; and (c) cell surface IgG reactivity on monkey esophagus epithelium by indirect immunofluorescence. The clinical and immunoserological characteristics of the patients are shown in Table 1. Except for patient 2, who also had Dsg1-reactive IgG, all of the other patients predominantly demonstrated IgG reactivity to Dsc3. Figure 1 depicts representative clinical pictures of the studied patients. All patients gave written consent to participate in this investigation, which adhered to the Declaration of Helsinki Guidelines and was approved by the local ethics committee.

Recombinant Dsc and Dsg Proteins

The recombinant Dsc and Dsg proteins were expressed in insect cells (High Five; Invitrogen) by infection with recombinant baculovirus as previously described[9,15] (Figure 2A). Culture supernatants of infected cells were collected after 3 days and recombinant proteins were purified by affinity chromatography using nickel-nitrilotriacetic-linked agarose beads (Quiagen) according to the manufacturer's instructions. Purified proteins were gradually dialyzed against phosphate-buffered saline (PBS) supplemented with 0.5 mmol/L $CaCl_2$ and stored at −20°C. By immunoblot analysis, the purified recombinant proteins displayed the expected size and were specifically immunoreactive using a monoclonal mouse anti-Etag antibody (1:1000; Amersham) (Figure 2B, upper panel). In addition, serum of a patient with paraneoplastic pemphigus showed reactivity with recombinant Dsc 1-3, Dsg 1, and Dsg3 protein (Figure 2B, lower panel).

Purification of Dsc3-Reactive IgG from Pemphigus Sera

Affinity chromatography columns were prepared with CNBr-activated sepharose 4B (Amersham Pharmacia Biotech) diluted in 1 mmol/L HCl as recommended by the manufacturer. Before coupling, the ligands (recombinant

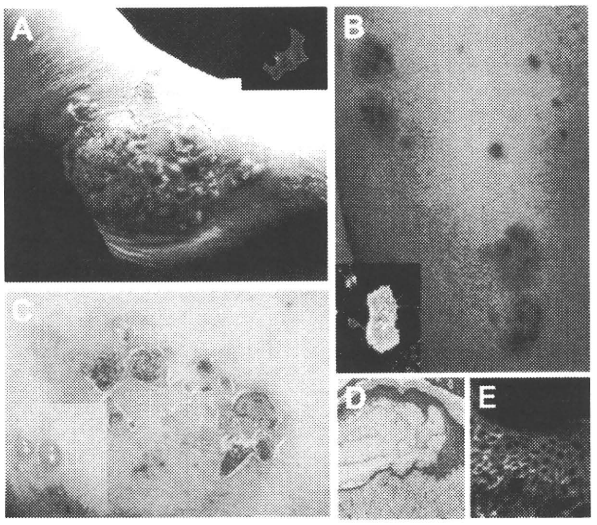

Figure 1. Clinical phenotype of representative patients with atypical pemphigus. Clinical pictures of two representative patients with atypical pemphigus (pemphigus vegetans and pemphigus herpetiformis), that illustrates hypertrophic verrucous plaques with pustules and erosions of the foot (**A**) and multiple erythematous herpetiform erosions of the trunk and extremities (**B**, **C**). Insets of **A** and **B** show IgG reactivity of the patients' sera with human desmocollin 3-transfected COS-7 cells. Histopathology of lesional skin reveals intraepidermal loss of keratinocyte adhesion at the suprabasilar level (**D**). Direct immunofluorescence shows IgG deposits on the surface of epidermal keratinocytes (**E**).

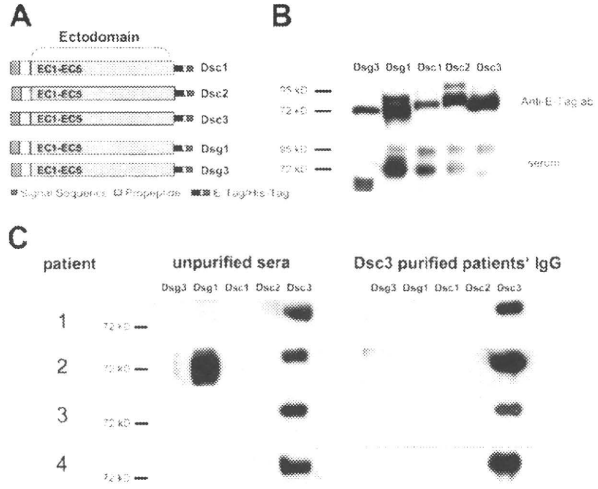

Figure 2. Affinity purification of desmocollin 3 (Dsc3)-reactive IgG autoantibodies from four patients with atypical pemphigus. **A:** Scheme of the recombinant proteins applied in this study, ie, the entire ectodomains of Dsc1, Dsc2, Dsc3, desmoglein 1 (Dsg1), and desmoglein 3 (Dsg3) linked to E- and 6xHis-Tag. **B:** Detection of the recombinant proteins by immunoblot analysis using an anti-E-tag monoclonal antibody (**upper panel**) or serum of a patient with paraneoplastic pemphigus (**lower panel**). **C:** Immunoblot analysis revealing IgG reactivity of the four atypical pemphigus sera, with the recombinant proteins before (**left panel**) and after (**right panel**) affinity purification, using Dsc3 columns. Patient 2 shows additional IgG reactivity with Dsg1.

Dsc3 or Dsg3) were dialyzed against coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and incubated with the sepharose medium overnight at 4°C. The next day, the coupled sepharose was subjected to five washes with coupling buffer followed by incubation with blocking buffer (0.1 M Tris-HCl at pH 8.0) for 2 hours at room temperature. The coupled sepharose was then washed four times with 0.1 M acetate and 0.5 M NaCl at pH 4.0 and four times with 0.1M Tris-HCl and 0.5 M NaCl at pH 8.0 alternately. Finally, the sepharose was washed with PBS containing 0.5 mmol/L $CaCl_2$ at pH 7.4.

Pemphigus sera were diluted at 1:5 in PBS containing 0.5 mmol/L $CaCl_2$ at pH 7.4. The Dsc3 column was incubated with the diluted sera overnight at 4°C. The next day, the pre-adsorbed sera were collected and the column was washed three times with PBS containing 0.5 mmol/L $CaCl_2$ + 0.05% Tween 20 at pH 7.4 and, subsequently, three times with PBS containing 0.5 mmol/L $CaCl_2$ at pH 7.4. The antibodies were eluted using 100 mmol/L Glycin-HCl (pH 2.7) and 900 µL aliquots were collected in tubes containing 100 µL of 1 M Tris-HCl (pH 9.0). The antibodies containing aliquots were combined (total volume of 15 ml) and dialyzed overnight against PBS with 0.5 mmol/L $CaCl_2$ at pH 7.4. The purified IgG fraction was concentrated 10-fold using Amicon Ultra-15 Centrifugal Filter Units (Millipore). The protein concentration of the eluate was measured using a modified Lowry protein assay. Final protein concentrations ranged between 100 and 200 µg/ml.

Immunoblot Analysis with Dsc and Dsg Recombinants

The Dsg and Dsc recombinants were run on 10% SDS-PAGE and blotted onto nitrocellulose membranes as previously described.[9,15] Membranes were blocked with 5% milk powder in PBS, 0.05% Tween-20 (PBS-T) with 0.5 mmol/L $CaCl_2$. Pemphigus sera (1:100) and purified IgG fractions (1:20) were diluted in PBS-T with 0.5 mmol/L $CaCl_2$ and 5% milk powder and were incubated with the blotted membranes overnight at 4°C. After three washes with PBS-T supplemented with 0.5 mmol/L $CaCl_2$, the nitrocellulose membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-human IgG (1:5000; Dako) for 1 hour at room temperature. Specific immunoreactivity was then visualized using Immobilon Western Chemiluminescent HRP substrate (Millipore) (Figure 2, B and C).

Immunofluorescence Studies

The immortalized keratinocyte cell line HaCaT was cultured in Dulbecco's modified Eagle's medium (DMEM high glucose; Invitrogen) supplemented with 10% fetal calf serum (PAA), 50 units/ml penicillin-G, 50 µg streptomycin and 2 mmol/L L-Glutamine (Gibco). For immunofluorescence staining, HaCaT cells were grown subconfluent in chamber slides (Nunc/Thermo, Fisher Scientific) using epidermal keratinocyte medium (CnT-57 medium; CELLnTEC Advanced Cell Systems). Subconfluent keratinocyte cultures were switched to defined keratinocyte medium (CnT-02 medium; CELLnTEC Advanced Cell Systems) supplemented with 1.2 mmol/L $CaCl_2$ 24 hours before adding the IgG fractions. Keratinocytes were then incubated for 2 hours at 4°C with purified anti-Dsc3 IgG diluted at 1:2, pooled IgG from healthy donors 1:2 diluted or anti-Dsc3 monoclonal antibodies (clone U114, 1 µg/ml). After three washes with PBS containing 1.2 mmol/L $CaCl_2$ and 1.0 mmol/L $MgCl_2$ $6H_2O$, keratinocytes were fixed in 1% paraformaldehyde-PBS for 20 minutes at room temperature. After three washes, cells were incubated with PBS containing 0.5% Triton X-100 for 10 minutes at room temperature and were finally incubated with fluorescein isothiocyanate (FITC)-labeled anti-human IgG at 1:200 in 1% bovine serum albumin-PBS (dianova) (Figure 3A–F).

Frozen sections of normal human skin were blocked for 30 minutes with PBS supplemented with 10% normal goat serum. Samples were incubated for 1 hour at room temperature with purified Dsc3-reactive IgG (diluted in PBS with 2% goat serum at 1:2), IgG pooled from healthy donors at the same dilution or anti-Dsc3 monoclonal IgG (U114) as positive control for Dsc3 reactivity. After three washes with PBS, the skin sections were incubated with FITC-labeled rabbit anti-human IgG (1:200; dianova) for 30 minutes at room temperature (Figure 3G–L).

Indirect immunofluorescence on monkey esophagus was performed following a standardized protocol according to the manufacturer's instructions (The Binding Site). (Figure 3M–R).

Dispase-Based Keratinocyte Dissociation Assay

Primary human epidermal keratinocytes were seeded in 12-well plates and grown to confluence in CnT-57 medium (CELLnTEC Advanced Cell Systems) as previously described.[16] The day before the assay, cells were cul-

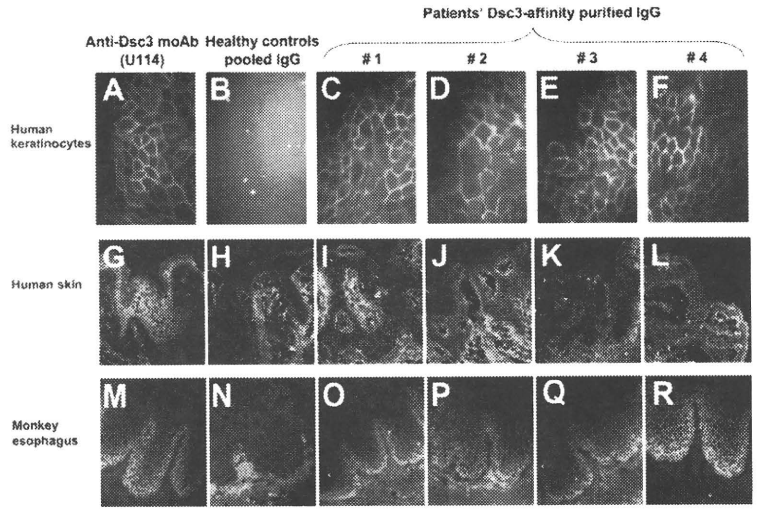

Figure 3. Reactivity of the purified desmocollin 3 (Dsc3)-reactive IgG autoantibodies with cultured human keratinocytes, human epidermis, and monkey esophagus. Dsc3 affinity-purified IgG from four patients, with atypical pemphigus, reveal intercellular reactivity with cultured human keratinocytes (HaCaT cells) (patients 1–4 [**C–F**]), human epidermis (**I–L**) and monkey esophagus (**O–R**). Monoclonal anti-Dsc3 antibody (clone U114) (**A**, **G**, and **M**) and pooled IgG of healthy control sera (**B**, **H**, and **N**) served as positive and negative controls, respectively.

tured in CnT-02 medium (CELLnTEC Advanced Cell Systems) containing 1.2 mmol/L $CaCl_2$ and incubated with purified Dsc3-reactive IgG (at 20 μg/ml), pooled control IgG (20 μg/ml) or anti-Dsc3 monoclonal antibody (U114 at 1 μg/ml), overnight at 37°C. Two hours before the assay termination, recombinant exfoliative toxin A (Toxin Technology) was added at 0.5 μg/ml to cleave Dsg1 on the keratinocyte surface. After two washes with PBS, the adherent keratinocytes were incubated at 37°C for 20 minutes with dispase I (Roche Applied Sciences) resulting in a non-adherent cell monolayer. The monolayers were carefully washed twice with PBS and subjected to mechanical stress by pipetting ten times with a 1-ml pipet. Fragments were fixed in 1 ml of a 10% formalin solution and stained with crystal violet. Images were captured using a digital camera and fragments were counted by five different blinded observers (Figure 4). Relative dissociation scores were calculated using the number of keratinocyte fragments in relation to the maximal number of cell fragments obtained by the positive control (anti-dsc3 monoclonal antibody, clone U114).

Results

Dsc3-Reactive Autoab in the Sera of Four Patients with Atypical Pemphigus

Sera of four patients with pemphigus herpetiformis and pemphigus vegetans, respectively, were included in this study. Patients presented with multiple erythematous herpetiform erosions and with hypertrophic verrucous plaques with pustules and erosions, respectively, which are illustrated in Figure 1. Histopathology analysis of lesional skin indicated intraepidermal loss of keratinocyte adhesion at the suprabasilar layer (Figure 1D). Furthermore, direct immunofluorescence revealed an intercellu-

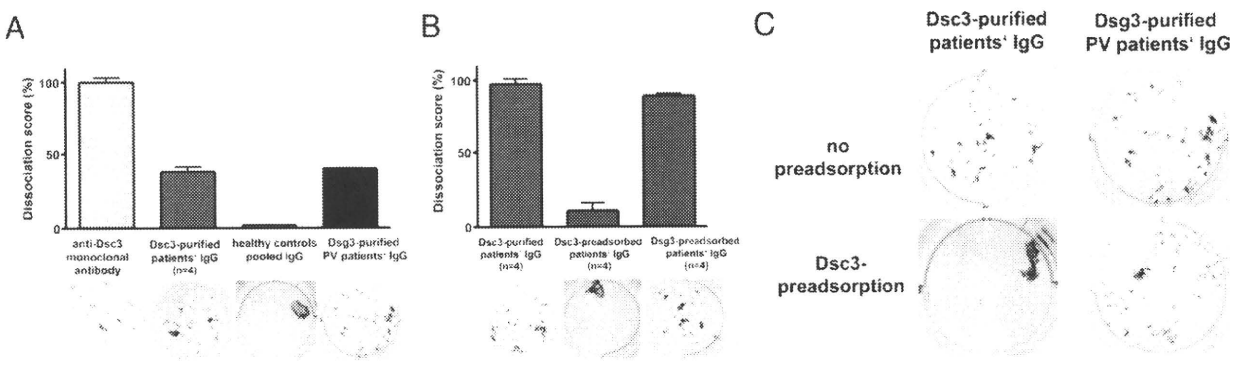

Figure 4. Desmocollin 3 (Dsc3)-affinity purified IgG from four patients with atypical pemphigus induces loss of keratinocyte adhesion. Results of the dispase-based keratinocyte dissociation assay using primary human epidermal keratinocytes are illustrated. Keratinocyte monolayers were incubated with the monoclonal anti-Dsc3 IgG antibody (clone U114), Dsc3-affinity purified IgG from the four atypical pemphigus patients, pooled IgG from healthy donors, or desmoglein 3 (Dsg3)-affinity purified IgG from a pemphigus vulgaris patient. **A:** Quantification of the keratinocyte fragments is depicted as a relative dissociation score. The maximal number of keratinocyte fragments obtained after incubation with the monoclonal anti-Dsc3 IgG antibody (clone U114) is set as 100%. Representative images out of three independent experiments are shown below the diagram. **B:** Number of keratinocyte fragments after incubation with Dsc3-affinity purified IgG from four atypical pemphigus patients is illustrated as a dissociation score of 100% (**B**, **left column**). The induction of keratinocyte dissociation is Dsc3-specific because pre-adsorption of the Dsc3-affinity purified IgG fraction with recombinant Dsc3 (**B**, **middle column** and **C**, **left panel**) but not with human Dsg3 (**B**, **right column**) blocks keratinocyte dissociation. To exclude non-specific binding of IgG to recombinant Dsc3 protein, Dsg3-purified IgG from a pemphigus vulgaris patient was pre-adsorbed with Dsc3 protein (**C**, **right panel**). Dsg3 affinity purified IgG induces loss of keratinocyte adhesion (**A** and **C**) that is not inhibited by pre-adsorption with human Dsc3 protein (**C**, **right panel**).

lar staining pattern, ie, positive IgG deposits on the surface of epidermal keratinocytes (Figure 1E).

To identify the desmosomal target proteins recognized by the IgG autoab of these patients, the sera were reacted with defined baculovirus-derived proteins including Dsg1, Dsg3, and Dsc1-3[9,15] (Figure 2A, B upper panel). Immunoblot analysis revealed the presence of IgG against Dsc3 in all of the studied patients; in addition, the serum of patient 2 showed IgG reactivity against Dsg1 (Figure 2C, left panel; Table 1).

Dsc3-Specific IgG Recognizes Recombinant and Native Dsc3

Serum samples of four atypical pemphigus patients were affinity purified using a Dsc3-coupled-CNBr sepharose column to obtain and further analyze the Dsc3-reactive IgG fraction. After purification, the IgG fractions of the four studied patients reacted exclusively with recombinant Dsc3, which was indicated by immunoblot analysis (Figure 2C, right panels). To determine the reactivity of the Dsc3-purified IgG also with native Dsc3, immunofluorescence of cultured human keratinocytes (HaCat) and human skin samples was also performed (Figure 3). The anti-Dsc3 monoclonal antibody U114 was used as a positive control and showed intercellular staining of both keratinocyte cell surface (Figure 3A) and human epidermis (Figure 3G), whereas pooled IgG from healthy control sera did not show any specific staining (negative control, Figure 3B, H). The Dsc3-purified IgG fractions of patients 1–4 demonstrated intercellular reactivity with cultured human keratinocytes (Figure 3C–F) and human epidermis (Figure 3I–L). In addition, indirect immunofluorescence was performed on monkey esophagus confirming the previous results (Figure 3M–R). The anti-Dsc3 monoclonal antibody, U114, elicited an intercellular staining pattern predominantly at the expected basal and suprabasal levels of the mucosal epithelium (Figure 3M). Very similar results were obtained subjecting the Dsc3-purified IgG fractions of patients 1–4 to immunofluorescent staining on monkey esophagus (Figure 3O–R), demonstrating the binding of these Dsc3-purified autoab to native Dsc3.

Dsc3-Specific IgG Induces Loss of Keratinocyte Adhesion In Vitro

After confirming the reactivity of Dsc3-purified IgG autoab with recombinant and native Dsc3, we sought to investigate the pathogenic relevance of these autoab. Interference with keratinocyte adhesion is considered the pathogenic hallmark of autoab in pemphigus. Thus, we applied a well established *in vitro* system for evaluating the capability of Dsc3-purified IgG autoab to induce loss of keratinocyte adhesion.[16] Dsc3-purified IgG of pemphigus patients 1–4 was applied to a dispase-based keratinocyte dissociation assay using primary human keratinocytes. Confluently grown primary keratinocytes were incubated with Dsc3-purified IgG fractions of patients 1–4, and afterward Dsg1 was degraded by incubation with exfoliatoxin A. Next, mechanical stress was applied to the keratinocyte monolayer to obtain cellular fragments illustrating the loss of keratinocyte adhesion in these keratinocyte cultures and thus, the pathogenicity of the Dsc3 autoab studied. Figure 4 illustrates that the anti-Dsc3 monoclonal antibody, U114, induces a dramatic weakening of keratinocyte adhesion, leading to a high number of keratinocyte fragments, whereas control IgG from pooled healthy individuals does not affect keratinocyte adhesion (Figure 4A). Keratinocyte cell sheets incubated with Dsc3-purified IgG were dissociated into numerous smaller fragments, indicating that Dsc3-reactive IgG of patients' sera is capable of inducing loss of keratinocyte adhesion (Figure 4A).

Furthermore, it was crucial to investigate whether this effect on keratinocyte adhesion was specific for the Dsc3-reactive autoab and related to the presence of Dsc3 in desmosomes. Pre-adsorption of the Dsc3-purified IgG fractions with recombinant Dsc3, but not with recombinant Dsg3, completely blocked the loss of keratinocyte adhesion in the dissociation assay (Figure 4B). To exclude non-specific binding of IgG autoab to recombinant Dsc3, we also applied Dsg3-purified IgG from PV patients to the keratinocyte dissociation assay. As previously described, Dsg3-reactive autoab of a pemphigus vulgaris patient induces loss of keratinocyte adhesion in this assay (Figure 4, A and C). However, pre-incubation of anti-Dsg3 IgG with recombinant Dsc3protein did not abrogate this effect (Figure 4C right panel). Thus, these results strongly suggest that the Dsc3-purified IgG fraction in the atypical pemphigus patients studied here is pathogenic and induces loss of keratinocyte adhesion by specifically binding and/or blocking Dsc3 and such effect can be blocked by preincubation with the recombinant Dsc3-protein, but not with Dsg3.

Discussion

This study presents that Dsc3-reactive autoab from patients with atypical pemphigus induce loss of epidermal keratinocyte adhesion. The affinity-purified Dsc3 autoab from the four atypical pemphigus patients induced loss of cellular adhesion in a dispase-based keratinocyte dissociation assay. These results extend recent findings of Spindler et al,[14] which identified Dsc3 as a crucial component of desmosomal adhesion. Moreover, they reported that epidermal adhesion mediated by Dsc3 is inhibited by sera from a subset of patients with atypical pemphigus.[14] IgG autoab against Dsc3 are not exclusively associated with atypical pemphigus; we and others have also identified IgG reactivity against Dsg1 and Dsg3 in single cases of pemphigus herpetiformis.[17,18]

The best evidence that Dsc3 is critically involved in desmosomal adhesion in the epidermis comes from a recently established Dsc3$^{-/-}$ mouse model.[13] The Dsc3$^{-/-}$ mice revealed an impressive phenotype with extensive intraepidermal blistering and telogen hair loss. This phenotype shares some similarities with a mouse deficient of Dsg3, the autoantigen of PV.[19] Thus, in addition to Dsg3 and Dsg1, Dsc3 must be considered as a relevant component for desmosomal adhesion of epidermal keratinocytes.

Until now, there was only circumstantial evidence for a pathogenic role of IgG autoab against distinct Dsc isoforms.

Dsc1 has been previously identified as a target antigen of IgA autoab in the subcorneal pustular dermatosis type of IgA pemphigus.[8,20] Except for the findings of a single *in vitro* study,[21] there is currently no clear evidence for the pathogenicity of IgA autoab on desmosomal cell-cell adhesion. Autoab against Dsc of the IgG and IgA classes have been occasionally identified in patients with atypical pemphigus[9,10–12,17] and paraneoplastic pemphigus.[9,22,23]

It is currently a matter of debate whether IgG or IgA autoab against Dsc are rare phenomena in pemphigus or whether their detection is limited by the diagnostic tools that are currently available. Hisamatsu et al[11] did not detect IgG or IgA autoab against Dsc1-3 in 45 sera from classical pemphigus patients by enzyme-linked immunosorbent assay (ELISA) with recombinant proteins. The sensitivity of the Dsc-ELISA was limited because all of the sera from eight patients with IgA pemphigus revealed IgA reactivity with Dsc1 expressed on COS cells, but only one serum was IgA positive by ELISA.[11] Using baculovirus-derived, eukaryotic recombinants of Dsc1, Dsc2, and Dsc3, Müller et al[9] were not able to detect Dsc-specific IgG in the sera of a cohort of 74 European patients with PV by ELISA and immunoblot, respectively. However, a considerable number of sera from patients with atypical forms of pemphigus, paraneoplastic pemphigus, and IgA pemphigus demonstrated either IgG or IgA reactivity with Dsc1, Dsc2, or Dsc3.[9] This observation is also reflected by the findings of the present study that the four Dsc3-reactive sera studied here were derived from patients with atypical pemphigus (pemphigus vegetans and pemphigus herpetiformis) who presented with cutaneous blisters; additional IgG reactivity against Dsg1 was found in only one case. Thus, the low detection rate of Dsc-reactive IgG autoab in PV and PF is presumably not a technical artifact, rather it is due to the low prevalence of Dsc-reactive autoab in PV or PF.

In summary, the present findings demonstrate that IgG autoab against Dsc3 induce loss of keratinocyte adhesion, which strongly suggests their pathogenic relevance in pemphigus. Because Dsc3-reactive IgG is only rarely found in patients with classical PV or PF, the observed *in vitro* pathogenicity of anti-Dsc3 IgG autoab provides a sound explanation why Dsc3-reactive patients with atypical pemphigus lacking IgG against Dsg3 or Dsg1 develop skin blistering.

Acknowledgments

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Neural System Antigens Are Recognized by Autoantibodies from Patients Affected by a New Variant of Endemic Pemphigus Foliaceus in Colombia

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Abstract
Background Endemic pemphigus foliaceus (EPF), is also known as "fogo selvagem" or "wild fire," reflecting the intense burning sensation of the skin reported by patients with this disease. Based on this finding, we tested for neural autoreactivity in patients affected by a new variant of EPF (El Bagre-EPF).
Methods We tested 20 El Bagre-EPF patients, 20 normal controls from the endemic area, and 20 age- and sex-matched normal controls from outside the endemic area. We tested for autoreactivity to several immunoglobulins and complement. Both human skin and bovine tail were used as antigens.
Results We detected autoreactivity to neural structures, mechanoreceptors, nerves, perineural cell layers of the arachnoid envelope around the optic nerve, brain structures, and to neuromuscular spindles; these structures colocalized with several neural markers. The patient antibodies also colocalized with desmoplakins 1 and 2, with the armadillo repeat protein deleted in velo-cardio-facial syndrome and with p0071 antibodies. Autoreactivity was also found associated with neurovascular bundles innervating the skin, and immunoelectron microscopy using protein A gold against patient antibodies was positive against the nerve axons. Paucicellularity of the intraepidermal nerve endings and defragmentation of the neural plexus were seen in 70% of the cases and not in the controls from the endemic area ($p<0.005$). Neuropsychological and/or behavioral symptoms were detected in individuals from the endemic area, including sensorimotor axonal neuropathy.
Conclusions Our findings may explain for the first time the "pose of pemphigus," representing a dorsiflexural posture seen in EPF patients vis-a-vis the weakness of the extensor nerves, and furthermore, the autoreactivity to nerves in EPF could explain the "burning sensation" encountered in EPF disease.

Keywords Autoantibodies · mechanoreceptor · pacinian corpuscle · nerves · endemic pemphigus foliaceus · epidermal nerve fiber density · myelin basic protein · glial fibrillary acidic protein · CD57 · protein gene product 9.5 · desmoplakins · optic nerve · arachnoid envelope antibodies

Abbreviations

EPF	Endemic pemphigus foliaceus
El Bagre-EPF	endemic pemphigus foliaceus in El Bagre, Colombia
FS	fogo selvagem
ICS	intercellular stain between the keratynocytes
BMZ	basement membrane zone
DIF and IIF	direct and indirect immunofluorescence

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ENFD	epidermal nerve fiber density
SDI	silver diamine ion stain
BP	bullous pemphigoid
MBS	modified Bielschowsky stain
NHP	Nottingham Health Profile
LKS	Lysholm Knee Scale
NDIC	Normarski differential interference contrast confocal microscopy
IHC	immunohistochemistry
CFM	confocal microscopy
IB	immunoblotting
IEM	immunoelectron microscopy
ARVCF	armadillo repeat protein deleted in velo-cardio-facial syndrome

Introduction

We have identified a new variant of EPF in El Bagre, Colombia, S.A. (El Bagre-EPF) [1–8]. Our studies established that this focus exhibits alike features to those described in Senear–Usher syndrome, a disease with combined features of lupus erythematosus and pemphigus [1–8]. This new variant of El Bagre-EPF differs from classic endemic pemphigus foliaceus (EPF) in aspects such as the following: (1) no clinical involvement of children or persons under the age of 30 years occurs, contrary to fogo selvagem (FS) that primarily affects children and young adults, with the highest incidence at 10–30 years of age, and both sexes equally affected; (2) El Bagre-EPF affects predominantly 40- to 60-year-old men, as well as a few post-menopausal women, in contradistinction to EPF; and (3) the main autoantigens are plakins (desmoplakins, envoplakin, periplakin, and plakoglobin), in comparison to EPF, where most autoantigens are desmogleins1 [1–8]. Both EPF as well as in El Bagre-EPF patients have intercellular deposits of immunoglobulins and/or complement showing intercellular stain (ICS) between the keratynocytes, but contrary to El Bagre-EPF, EPF does not exhibit deposits of antibodies and complement at the basement membrane zone (BMZ) of the skin [1–12].

EPF is also known by the name "fogo selvagem" or "wild fire" because patients affected by this illness classically report a burning sensation on the skin [1–12]. Two main questions about EPF remain unknown over almost a century: what explains the burning sensation of the patients' skin, which represents the hallmark symptom of this disease, and what explains the muscular weakness and the "pose of pemphigus" (a dorsiflexural position of the body), present in most chronically affected patients before the steroid era [1–12].

The population in El Bagre is exposed to tropical diseases, as well as to mercury and other pollutants as a result of gold mining and agricultural chores [3]. We reported the presence of mercury in skin biopsies from people living in the endemic area [3]. Given the burning sensation present in El Bagre-EPF and FS patients and noting that mercury is a neurotoxin, we searched for nerve alterations by hematoxylin and eosin (H&E), silver diamine ion (SDI), and modified Bielschowsky (MBS) staining. We performed direct and indirect immunofluorescence (DIF and IIF), immunohistochemistry (IHC), confocal microscopy (CFM), immunoblotting (IB), and immunoelectron microscopy (IEM) to study patient autoreactivity to several neural components.

Materials and Methods

We examined 20 El Bagre-EPF patients, 20 normal controls from the endemic area, and 20 normal controls from outside of the endemic area (all matched by age, sex, and work activity). In addition, we tested five patient samples of people suffering FS. El Bagre-EPF disease was characterized by epidemiological, immunological, histopathological, and clinical criteria reported elsewhere [1–3]. Simultaneously, we tested for neuropsychological, behavioral, and kinesiological alterations in the subjects of the study (all tested negative for *Mycobacterium leprae*). All cases displayed IgG4 intercellular staining between keratinocytes [1–3]. Sera of the cases were reactive to desmoglein 1 (Dsg1) by IB, by immunoprecipitation, and by enzyme-linked immunosorbent assay [1–3, 8]. In addition, in IB of the El Bagre-EPF, most sera reacted with plakins [including desmoplakins 1 and 2 (DPI and DPII), envoplakin, periplakin, plakoblobulin], to bullous pemphigoid (BP) antigens 1 and 2 and to other undetermined molecules [8]. For IIF, we used as substrates human chest skin, rat, mouse, and chipmunk nerve and brain tissue, and monkey esophagus (Oregon Primate Research Center, Portland, Oregon, USA). Selected samples for DIF and IIF were partially fixed in 3% paraformaldehyde [9, 10]. All samples were tested anonymously to comply with institutional review board requirements.

DIF and IIF Slides were incubated with the sera at 1:20–1:40 dilutions in phosphate-buffered saline (PBS), rinsed, and then incubated with secondary antibodies [including Fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG, IgM, IgA, IgD, IgE, C1q, C3c, C3d, albumin, and fibrinogen (all from Dako, Carpinteria, CA, USA)]. We also utilized anti-human total IgG/IgM/IgA antiserum (Zymed®; Invitrogen, Carlsbad, CA, USA) and rhodamine-conjugated goat anti-human IgA/IgM/IgG anti-

serum (Rockland Labs, Rockland, ME, USA). Neural marker colocalization was studied by utilizing anti-human S-100 antibody (Dako), mouse anti-human neuron specific enolase (NSE) (Dako), and Cy3 conjugated anti-glial fibrillary acidic protein (GFAP), (Sigma Aldrich, St. Louis, MO, USA). In addition, we used a multi-epitope cocktail containing anti-desmoplakin I and II (DPI and DPII), anti-armadillo repeat protein deleted in velo-cardio-facial syndrome (anti-ARVCF) and anti-p0071 antibodies (all from Progen). The secondary antibodies were conjugated with Texas Red and/or with Alexa 555 (both from Invitrogen). All sections were then examined with a Nikon Eclipse 50i microscope (Japan). All slides were counterstained with Dapi (Pierce, Rockford, IL, USA) or TO-PRO®-3/DNA (Invitrogen).

IHC Immunohistochemistry was used to study the colocalization of patient's autoantibodies with nerves, employing a dual endogenous peroxidase blockage, with the addition of an Envision dual link (Dako). We tested for anti-human IgG, IgM, IgA, IgD, IgE, C1q, C3c, C3d, albumin, and fibrinogen (all from Dako). We also utilized anti-S-100 proteins, GFAP, NSE, and rabbit anti-human myelin basic protein (MBP), anti-neurofilament, CD57, and protein gene product 9.5 (PPG 9.5) (all from Dako).

SDI and Modified MBS Samples were prepared as previously described [9], and all biopsies were taken from the chest area for accurate morphometric correlation [9–14]. Nerve density was determined by the average number of positive silver staining fibers in five low power fields under a light microscope [9, 10]. We quantified both the epidermal nerve fiber density (ENFD) and myelinated nerve endings per square millimeter [9–14].

Autoreactivity to Optic Nerve and Brain Structures To define autoreactivty of the El Bagre-EPF patients, we assessed immunoreactivity utilizing optic nerve and brain from rat, mouse, and chipmunk sources, and we performed colocalization with DPI, DPII, ARVCF, and p0071 antibodies.

Colocalization of El Bagre-EPF Autoantibodies and Neural Markers Utilizing CFM We performed CFM examinations using standard ×20 and ×40 objective lenses; each photoframe included an area of approximately 440×330 μm. Images were obtained using EZ 1 image analysis software (Nikon, Japan).

Immunoblotting IB testing for reactivity of IgM and IgG antibodies to bovine tail nerve was performed. The samples were extracted with 0.6 M Tris–HCl, pH 6.8, containing 2% sodium dodecyl sulfate (SDS), 5% ß-mercaptoethanol, and 1 mM EDTA, with 1 mM dithiothreitol and protease inhibitors. Extracts were run on a 10% SDS gel. The proteins were transferred to nitrocellulose membranes, and the immune reactions were carried out with patient and control sera. Chemiluminescence detection performed using an ECL kit including SuperSignal® West Pico Substrate ECL™ (Pierce, Rockford, IL, USA).

Neurological, Neuropsychological, Behavioral, and Kinesiological Testing Clinical studies were performed to identify neurological alterations, including autonomic neuropathies [15–17]. Numbness, paresthesias, urinary incontinence, sensitivity to pressure, cold, heat and pain, bedside orthostatic hypotension, a Valsalva ratio, beat-to-beat blood pressure, pupillary responses, and cranial nerves II–XII were assessed.

Kinesiology Evaluation We tested for kinesiological movement and skin shriveling in response to immersing the hand in hot or cold water and also utilized the Nottingham Health Profile (NHP) for the evaluation of muscular imbalance or derangement [11–17]. We used the Lysholm Knee Scale (LKS) to study limp support, impaired stair climbing, squatting, walking, running, jumping, atrophy of the thigh, pain, and swelling [16, 17]. All these parameters were rated as none, very mild, moderate, or severe.

ENFD, Nerve Morphometry, and Histomorphometric Analysis SDI, MBS, and IHC stains were used on the skin biopsies to quantify the density of intraepidermal axons, as well as the ENFD [12–15].

Immunoelectron Microscopy Postembedding immunogold labeling was performed on samples of El Bagre-EPF sera and controls. Peripheral rat nerve was used as an antigen; the tissue was dissected, fixed in 4% glutaraldehyde with 0.2% paraformaldehyde, and embedded in lowicryl® resin. The tissue was then sectioned at 70 nm thickness. The samples were blocked with a solution from Aurion™, Electron Microcopy Sciences (EMS, Hatfield, PA, USA). The grids were then washed with PBS-BSAC (Aurion™, EMS). The primary antibodies were incubated overnight at 4°C. The next day the grids were washed, and a secondary antibody solution, 10 nm gold-conjugated protein A PBS-BSAC (Aurion, EMS™) was applied. The samples were double-stained with uranyl acetate and lead citrate. The samples were observed under a Hitachi H7500 transmission electron microscope. Immunogold particles displaying any pattern of positivity were converted to TIF format.

Mercury Detection in Hair and Nails We measured mercury in the hair in all of the cases and controls. After hair washing, 25 mg of hair and/or nails was cut and degreased with acetone. Hair and nail samples were packed separately in ash-free paper and analyzed separately.

Samples were measured following methods described elsewhere [3]. Mercury was measured in an atomic absorption spectrophotometer (MAS 50). The World Health Organization defines a permissible level of mercury in hair of 7 ppm. We scale the results as negative (−) >7 ppm, weak positive >7–14 ppm (+), positive >14–28 ppm (++), very positive from 28>to 56 ppm (strong positive) (+++), and super strong (>56–144 ppm and upper) (++++).

Statistical Methods ENFD and myelinated nerve endings per square millimeter were statistically analyzed using the nonparametric Spearman's rank correlation, specifically with a p value of 0.05 and a single-tailed analysis. We determined that our data followed a normal distribution using the Kolmogorov–Smirnov test and used Student's t test to evaluate differences in morphology.

Results

All El Bagre-EPF patients and the five FS patients have a skin burning sensation, and no control from the endemic or non endemic area has this symptom ($p>0.005$).

Nerve Paucicellularity (Free Ending and Thin Skin Myelinated Nerves) Was Found in Most El Bagre-EPF Patients We detected reduced ENFD and decrease myelinated nerve fiber density in 70% ($p>0.005$) of the El Bagre-EPF patients and in three of five FS patients by the H&E, IHC, SDI, and MBS stains. We noted damage to subepidermal neural plexus areas in the El Bagre-EPF patients and in three of five FS patients, featuring fragmentation of both myelinated and non-myelinated fibers as well as reduction of the innervations of skin appendices. These findings were noted in only 6% of the control patients from the endemic area and in none normal controls from outside the endemic area. When utilizing antibodies to PPG9.5, CD57, neurofilament, GFAP, NSE, S-100, and MBP, fragmentation of the subepidermal nerve plexus fibers was appreciated, and specific loss of nerve fibers ascending vertically into the epidermis was observed (Figs. 1, 2, 3, 4, and 5).

Autoreactivity to Neural Structures Colocalizing with Neural Markers We found autoreactivity to Pacinian corpuscles (PC), mechanoreceptors, nerves, neuromuscular spindles, and neurovascular packages in 70% of El Bagre-EPF and in three of five FS patients ($p<0.005$). No controls show this reactivity. We demonstrated colocalization of the autoreactivity with multiple neural markers, such CD57, neurofilament, PPG 9.5, CD57, neurofilament, PPG 9.5, GFAP, S-100, and MBP (Figs. 1, 2, 3, 4, and 5).

Autoreactivity to Optic Nerve and Brain Structures Reactivity to perineural cell layers of the arachnoid envelope surrounding the optic nerve was noted in 12 of 20 El Bagre-EPF patients, versus no controls ($p<0.005$). This reactivity was not seen in the FS patients. The reactivity colocalized exactly with the antibodies to DPI, DPII, and ARVCF. Colocalization was also present in nearby vessels with p0071 by both IIF and CFM ($p<0.005$). In 12 of 20 El Bagre-EPF patients and in one of 20 controls from the endemic area (a brother of one El Bagre-EPF patients), autoreactivity was noted surrounding brain cisternae, colocalizing with the arachnoid envelope of the optic nerve (mainly, but some reactivity was seen inside the nerve) and with the brain gyral and the sulci surfaces ($p<0.005$). This reactivity was not seen in the FS patients, neither in control out of the endemic area (Figs. 1, 2, 3, 4, and 5).

Neuropsychological and Behavioral Performance Seventy percent of the El Bagre-EPF and three of five FS patients displayed neurological alterations that were statistically significantly higher in comparison with the other controls (i.e., symptoms of depression, anxiety, insomnia, and restlessness; $p<0,005$). Intellectual sequelae (mainly alteration of executive function and constructional praxis) were not found to be different between patients and controls. Conversely, the kinesiological alterations and the burning sensation on the skin were present in 100% of the El Bagre-EPF and in three of five FS patients compared to none of the controls neither inside or outside the endemic area ($p<0.005$). Autonomic alterations were also higher in the El Bagre-EPF patients ($p<0.005$), including urinary incontinence, itching, burning sensations, esophageal dysmotility, and sphincter relaxation. Pupillary and other reflexes were normal, as well as testing for cranial nerves (II–XII), and non-statistical differences were found in comparison with the controls. Numbness, paresthesias, sensitivity to pressure, cold, heat and pain, bedside orthostatic hypotension, valsalva ratio, and beat-to-beat blood pressure show no differences either.

The NHP analysis showed higher depression among the El-Bagre-EPF patients (100%), in contrast to the controls from the endemic area (10%; $p<0.005$). Postural deformities in the flexor tonus (as well as joint ankylosis and muscular weakness) were present in 70% of the El Bagre-EPF and in three of five FS patients, which was statistically significant to controls (5%; $p<0.0005$).

Figure 1 shows a series of DIF, IIF, CFM, and IHC images, demonstrating by multiple techniques the autoreactivity of the El Bagre-EPF patient sera against PC and optic nerve envelope. Autoreactivity to PC in El Bagre-EPF patients is shown (×100 for Fig. 1a and ×400 for Fig. 1c, d). Figure 1a shows positive staining to PC using rhodamine-conjugated goat anti-human IgG/IgM/IgA antiserum (blue

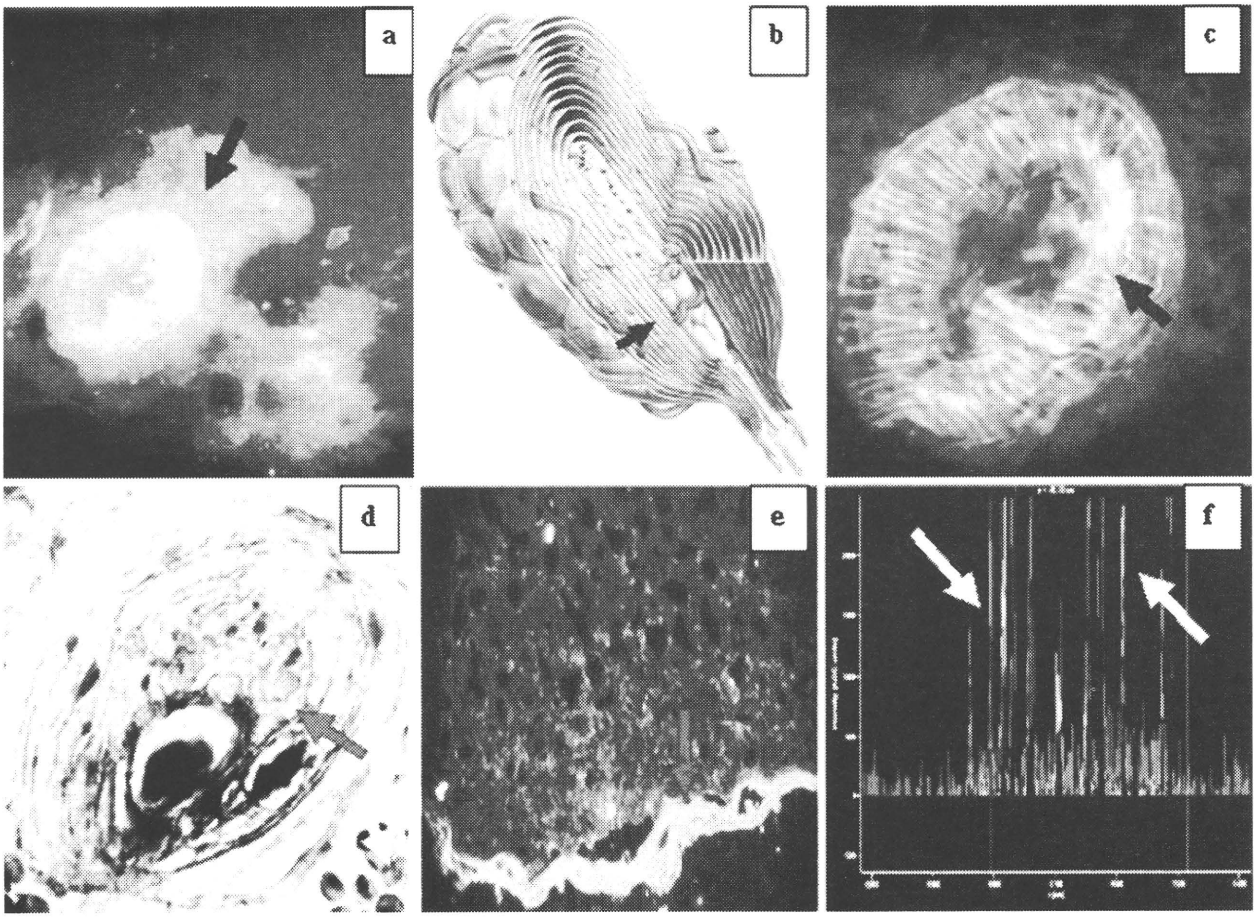

Fig. 1 A series of IIF, DIF, IHC, and NDIC images demonstrating by multiple techniques the autoreactivity of the El Bagre-EPF patient sera against the PC and optic nerves

arrow, orange stain). Figure 1b is a cover picture of a PC, reproduced with permission from SKF 26, and GDAS (Ratingen, Germany). Figure 1c is the higher magnification of Fig. 1a (×400; blue arrow), but in this case using anti-human IgG/IgM/IgA, conjugated with FITC (reactivity in green; blue arrow). Nomarski differential interference contrast confocal microscopy (NDIC) demonstrate that these structures were PC (data not shown). In Fig. 1c, IHC stains on El Bagre-EPF biopsies confirms the reactivity to PC (blue arrows) with anti-human IgM and IgA (dark brown stain onion shape stain, red arrow). Figure 1d is the IIF of a representative picture of a rat optic nerve, showing reactivity in the perineural meningeal sheaths recognized by El Bagre-EPF FITC conjugated anti-human IgG FITCI conjugated antibodies (red arrow, green staining). In some cases, delicate intra-optic nerve reactivity was also seen (not shown). No controls were positive. Using CFM, we were able to show that El Bagre-EPF patients reactivity colocalized with anti-P0071 antibodies (data not shown). In Fig. 1e, CFM shows a colocalization with antibodies to DP I and II (red panel) with the El Bagre-EPF autoantibodies (green panel). The blue peak is Dapi nuclear counterstain. The combined pikes colocalizing are pointed by the yellow arrows.

El Bagre-EPF Patient Sera Reactive Against Some Peripheral Nerves In Fig. 2 DIF, IIF, and IHC revealed autoreactivity against peripheral nerves. Figure 2a, c, d DIF reveals autoreactivity to peripheral nerve bundles, some directed against the epineururum, perineurum, and endoneurum. In Fig. 2a, the black arrows show positivity inside and in the periphery of the nerve with antibodies to IgG-FITCI conjugated from one El Bagre-EPF patient (yellowish-green stain); the red stain (blue arrows) is PP9.5 stain to show colocalization of El Bagre-EPF antibodies with neural components (Fig. 2b).El Bagre-EPF sera shows reactivity to peripheral nerves using FITC conjugated anti-human IgG/IgA/IgM polyclonal antibodies (black arrows) (green staining). Figure 2c is similar as Fig. 2a, but at ×100. Figure 2d shows H&E stain demonstrating a strong lymphohistiocytic infiltrate around one of the El Bagre-EPF dermal nerves and edema (blue arrows). Figure 2e shows IHC positive for

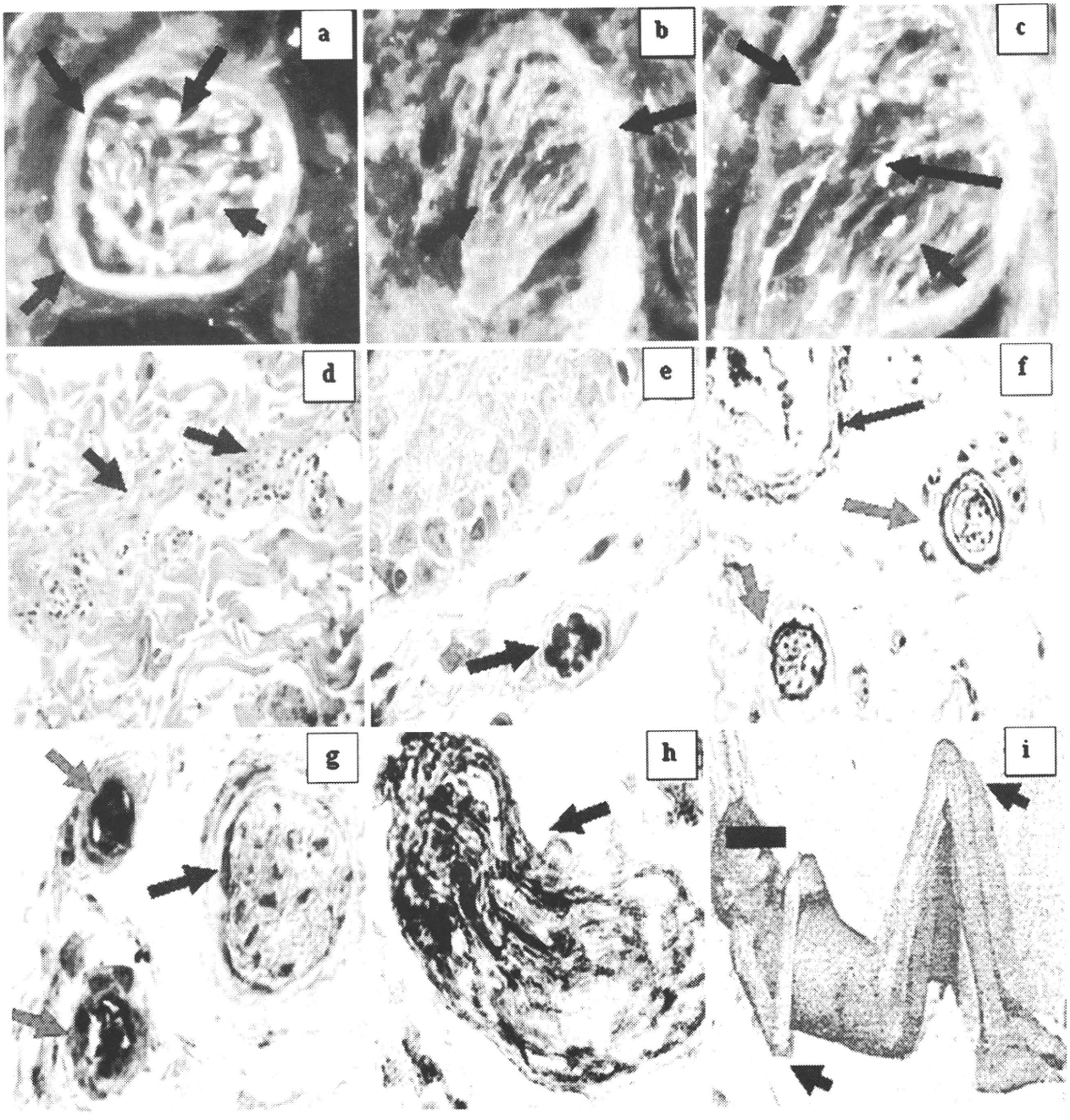

Fig. 2 A series of IIF, DIF, and IHC images demonstrating the autoreactivity of the El Bagre-EPF patient sera against several peripheral nerves

anti-human S-100 in nerves (blue arrow). Figure 2g shows nerve positivity with anti-human IgA (blue arrow) and positivity to two vessel (red arrows). Figure 2h demonstrates strong staining of a nerve with anti-human IgM antibody (blue arrow; brown staining). Figure 2i shows the "pose of pemphigus" with dorsiflexion of extremities (blue arrows). By IIF, 11 of the 20 El Bagre-EPF sera showed nerve reactivity to the thin nerves, especially to their epineurium, perineurium, endoneurium, and to vessels around and inside the nerve bundles. El Bagre-EPF patient antibodies colocalized with neural markers demonstrated by DIF, IIF, and IHC; positivity with S-100, NSE, PPG 9.5, GFAP, CD57, and neurofilament supports the neural origin of these antigens. The autoantibodies in El Bagre-EPF were polyclonal; thus, nine of the 20 samples tested positive to thin nerves utilizing anti-

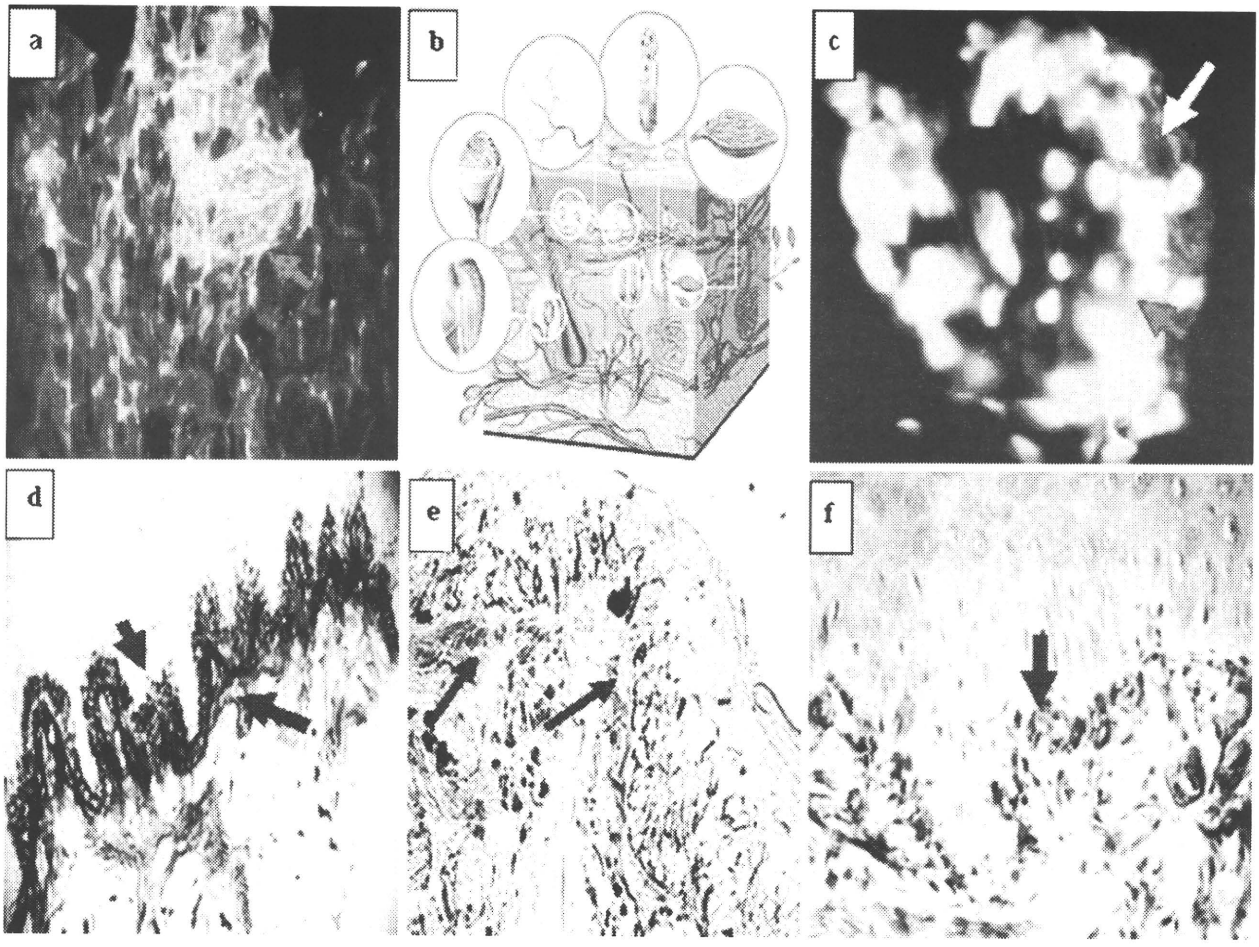

Fig. 3 El Bagre-EPF patient sera recognize other mechanoreceptors, and their skin biopsies show nerve paucicellularity and defragmentation of small nerves. "Pose of pemphigus"

human IgG/IgM/IgA antiserum. In contrast, two of the 20 normal controls from the endemic area showed autoreactivity to larger nerves with only IgM.

In Fig. 3, El Bagre-EPF patient sera recognize other mechanoreceptors, and their skin biopsies show nerve paucicellularity and defragmentation. Figure 3a shows positive staining of a Krause endbulb using FITC-conjugated anti-human IgM antibodies (red arrow) (green staining). Figure 3b is a diagram of mechanoreceptors of the skin. Figure 3c shows positivity with FITC-conjugated anti-human fibrinogen antiserum (red arrow, green staining), and with anti-human-ICAM-1/CD54 (orange staining, white arrow) in a Meissner corpuscle. The nuclei are counterstained with Dapi (blue). Figure 3d shows normal silver staining of the upper neural plexus of the skin of a normal control (dark brown netting stain; blue arrows). Figure 3e shows silver staining from one El Bagre-EPF skin biopsy, revealing defragmentation and loss of nerves (blue arrows). Figure 3f shows IHC of one El Bagre-EPF skin biopsy, utilizing anti-human C1q antibodies positive against the upper neurovascular bundle under the BMZ with anti-human C1q (blue arrow).

In Fig. 4. El Bagre-EPF patient sera recognize thin myelinated and non-myelinated nerves, the spindle cell apparatus, and brain tissue by different techniques. Figure 4a shows perineural inflammation and mild edema of a nerve in an H&E skin biopsy of an El Bagre-EPF patient (×100). Figure 4b and d is DIF showing positive colocalizations of El Bagre-EPF patient's autoantibodies using FITC conjugated-anti-human IgG antibodies (in Fig. 4b, to mechanoreceptors; in Fig. 4d, to the neuromuscular spindle; we used anti-GFAP to demonstrate the neural nature of these structures). The Bagre-EPF antibodies are shown in green staining (red arrows). In Fig. 4d, the red-orange stain shows El Bagre-EPF atutoreactivity to the neural spindle (yellow arrows). Figure 4c shows IB using cow spinal cord extract. On the left are the molecular weight standards and on the right is a positive control. After SDS polyacrylamide gel electrophoresis separation of cow spinal cord and blotting of neural substrate antigens, El