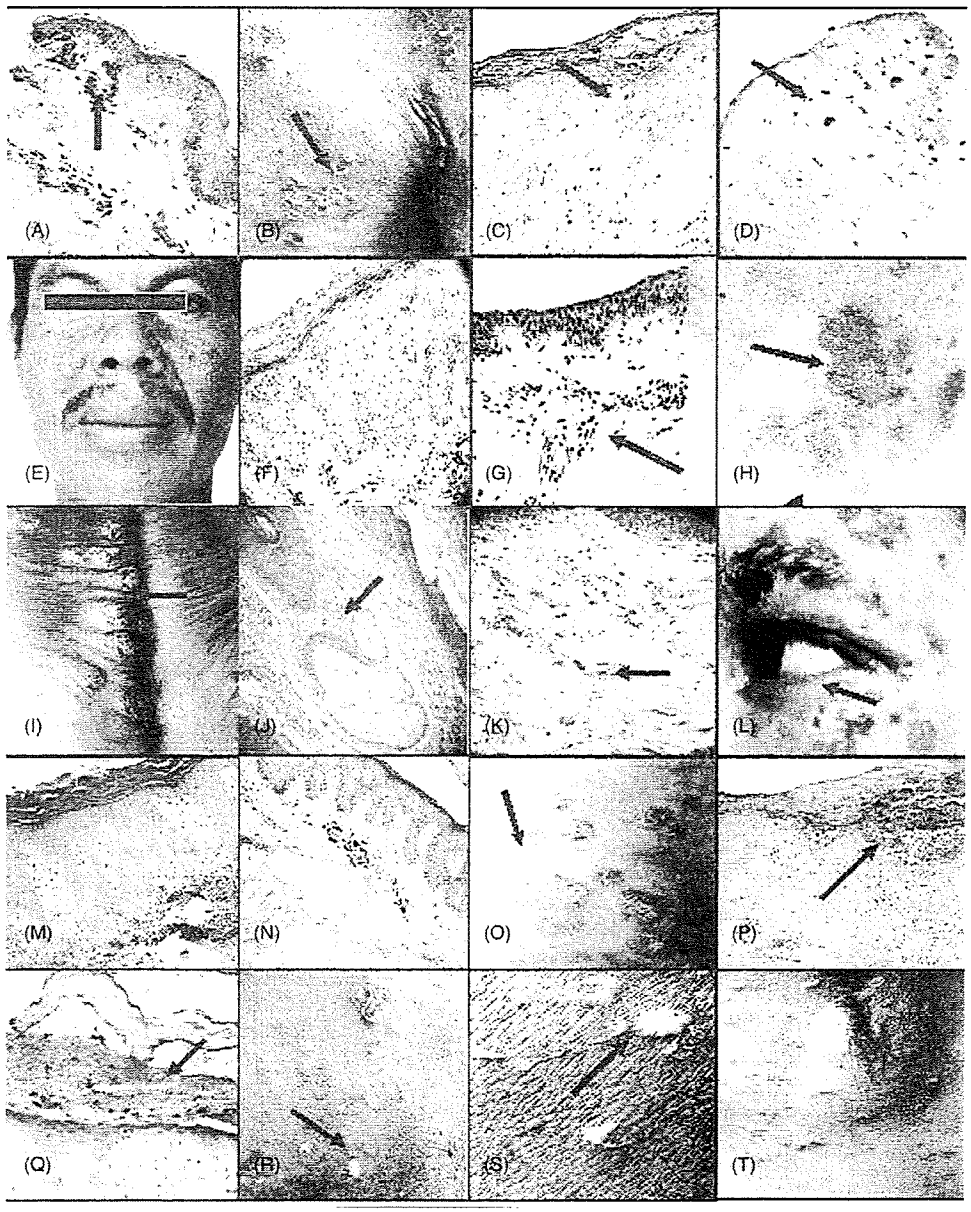

Fig. 1. Histopathologic patterns and their corresponding clinical lesions from patients affected by this new variant of endemic pemphigus foliaceus (EPF). A) Classical pemphigus foliaceus (PF)-like histopathology is appreciated, with subcorneal acantholysis and an inflammatory infiltrate surrounding the superficial vessels in the dermis. B) A series of bullae on the trunk with denudated areas and few pustules. C and D) Degeneration of the basal layer (C) and the atrophic epidermis (D). E) The face of this patient showed a butterfly rash, a scaling hyperkeratotic plaque on an erythematosus base. F and G) Regular, mild acanthosis and focal mild papillomatosis with numerous melanophages are also present within the papillary dermis. H and I) Crusts, hyperpigmented plaques and macules on the shoulder (H) and some papuloplaques around the axilla and torso (I). J and K) A patchy dermal perivascular infiltrate of lymphohistiocytic cells and melanophages is noticed, with some concomitant dilatation of blood vessels. Periappendageal infiltration of lymphohistiocytic cells, including the deep sebaceous glands, resembles focal histopathologic features seen in lupus erythematosus. L) A conjuctival ejection. M and N) A psoriasis-like pattern, with the rete ridges showing regular elongation but with variable thickening of the lower portions, in contradistinction to well-developed psoriasis in which uniform thickening in the lower rete ridges is often noticed. In some EPF cases, the dermal papillae are elongated and edematous, as often observed in psoriasis. In addition, scattered areas of parakeratosis were also seen in these biopsies from patients affected by EPF. O) Well-circumscribed bright red, circular papules and plaques with overlying silvery scale were noted on the trunk, resembling plaque-type psoriasis. P and Q) In contrast to psoriasis, large pustules beneath the cornified layer were common in some EPF patients, overlapping with classic histologic findings of a subcorneal pustular dermatosis. R and S) Note the large pustules on the chests of these patients. T) Axillary plaques of an El Bagre EPF patient, which were a very common clinical finding in this group of patients.

dry plaques resembling broad seborreic keratoses. These were mainly distributed under the axillare and sides of the chest (Fig. 1I).

Polymorphous light eruption-like lesions

In these cases, histologic characteristics included a superficial and deep dermal infiltrate of lymphocytes, with occasional eosinophils and neutrophils. Early cases might only show changes in the papillary dermis. Edema was often prominent in the upper dermis, and occasionally resulted in subepidermal bulla formation. The epidermis displayed varying degrees of spongiosis, with occasional parakeratosis and acanthosis (Fig. 1J,K). Interestingly, found these lesions typically presented on sun-exposed sites with an erythematous rash composed of micropapules. Several patients also displayed eyelid involvement, which correlates with recent data documenting immunohistologic compromise of eyelids and conjunctiva affected by El Bagre EPF (Abreu et al., manuscript in press in J Am Acad Dermatol) (Fig. 1L).

Psoriasis-like histologic features

These occurred in approximately 10% of the cases. Some features included focal epidermal psoriasiform changes, without thickening of the lower portion of the rete ridges (Fig. 1M,N). In a few cases, Kogoj spongiform pustules or Munro-like microabcesses were seen. Clinically, we observed well-demarcated, red-violet, round or circinate plaques in those patients (Fig. 1O). A symmetric distribution of skin lesions was often appreciated in these patients. In contrast to classic psoriasis, the clinical lesions of this group never involved the elbows, extensors surfaces, knees, intergluteal cleft, and genitalia; however lesions, similar to classic psoriasis, were occasionally present on the scalp, sacral and umbilical areas.

Subcorneal pustular dermatosis-like histologic features

In about 20% of the cases, histologic features resembled a subcorneal pustular dermatosis with vesiculopustules in the epidermis (Fig. 1P). Some microabscesses were seen within the corneal layer, while others were seen in subcorneal or granular cell layers. The subcorneal blisters were mostly filled with fibrinoid material (Fig. 1Q), and occasional erythrocytes without frank hemorrhage were observed. Clinically, these patients presented with pustular, mostly aseptic lesions as demonstrated by the previously described microbiologic studies. However, in contrast to lesions of classic subcorneal pustular dermatosis (Fig. 1R), the flexural aspects of the limbs were also involved except for the axillary regions (Fig. 1S,T).

A large percentage of patients (around 60%) from all El Bagre EPF cases present histologically with a combination of the above-mentioned patterns. For example, among patients with blisters, the most common blisters observed were subcorneal (40%), although in some cases subcorneal, intraspinous and subepidermal were visualized. In some cases, all of these blister patterns were noted simultaneously.

Although superinfected lesions where *Streptococcus* sp and *Staphylococcus* sp were cultured are not included in the current results, clinical lesions in these patients typically presented with ulcerated lesions with large crusts. The clinical presentation of these lesions was a burning sensation and excoriation of the lesions to ameliorate this symptom. Further, the burning sensation *per se* may be due to a healing process (Abreu et al., manuscript in preparation) or, alternatively, may be due to autoreactivity in dermal nerves detected by us in these patients (Abreu et al., manuscript in preparation).

Unfortunately, El Bagre EPF patients displayed no single, common histologic pattern. However, more acute cases have either acantholysis, or clefts or blisters at subcorneal, supraspinous, or BMZ areas. Mild cases and well-controlled chronic cases under prednisone treatment display only mild hyperkeratosis, consistent acanthosis and a large number of melanophages in the superficial dermis. In these chronic patients, the disease manifested clinical hyperpigmented macules and histologically, presence of melanophages surrounding superficial dermal vessels. The most severe cases displayed histologic features including an ulcerated epidermis extending into the deep dermis and involving cutaneous appendices, prominent serum scales, crusts and granulation tissue.

Palm findings

Glabrous palm skin showed some dermal inflammation without noticeable acantholysis or BMZ alterations by light microscopy (Fig. 2A,B). A similar pattern seen in all cases, included hyperkeratosis, hypergranulosis, edema in the papillary and reticular dermis (Fig. 2C-G) and some degree of dermal perivascular lymphocytic infiltration (Fig. 2C-E). DIF results for the palm biopsies will be reviewed in a separate publication (Abreu et al., manuscript in preparation).

IIF using monkey esophagus sections (Fig. 3)

Anti-IgG antiserum showed a weak epidermal cell surface stain with epidermal granular cytoplasmic pattern. A positivity within vessel walls in the upper dermis was also seen (5/5 cases). When anti-human IgA antiserum was used, 3/5 cases showed positive

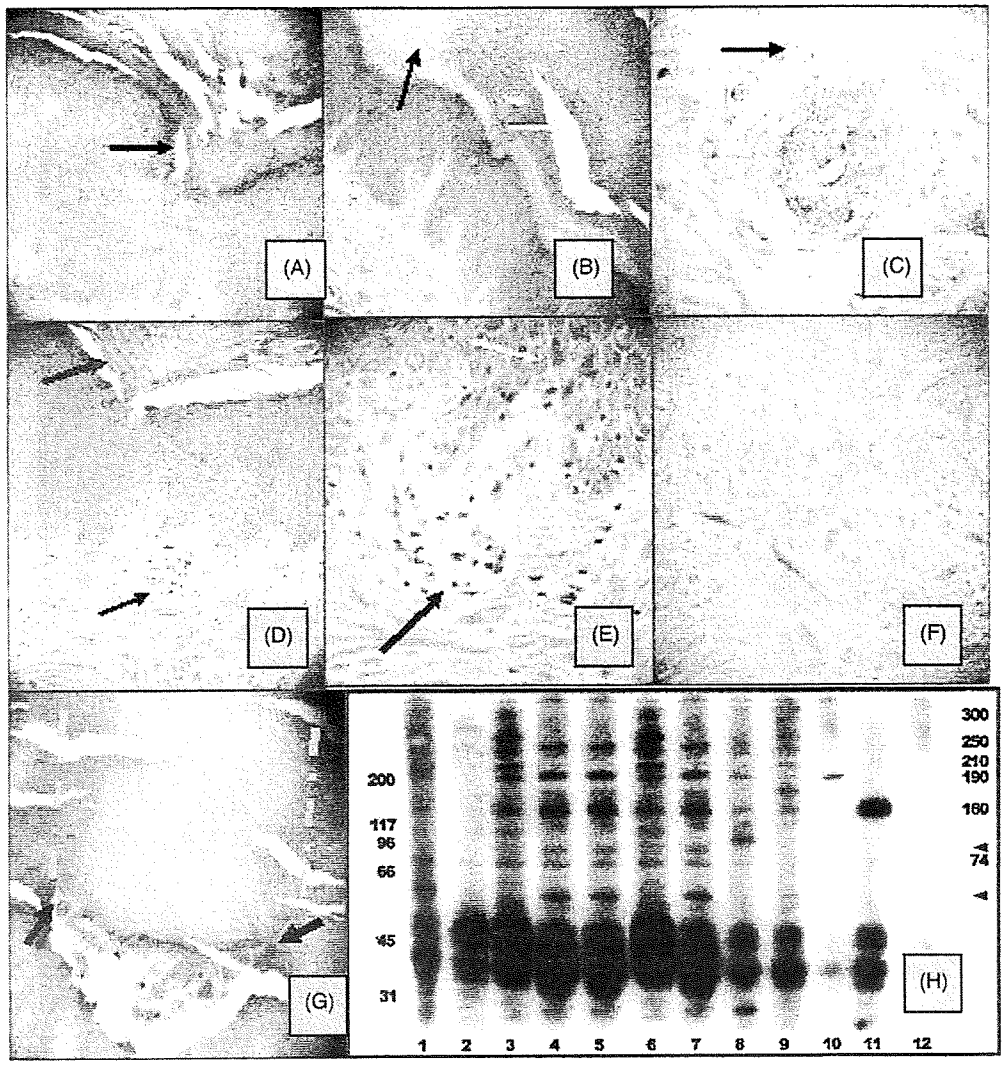

Fig. 2. (A) through (F) show hematoxylin and eosin (H&E) staining of biopsies from the palms of ten different patients affected with El Bagre endemic pemphigus foliaceus (EPF); these skin biopsies were taken from the patients simultaneously with other biopsies from non-glabrous skin. A) A small subcorneal separation (black arrow) (200x). B) Mild edema in the papillary dermis and thickened corneal layer on the palmar skin of some patients. Abnormality of the corneal layer, possibly resembling a disorder of keratinization (black arrow). Corneal layer separation may be because of fixation and/or processing artifacts? (yellow arrow) (200x). C) A lymphohistocytic perivascular infiltrate surrounding deep dermal vessels (black arrow) and thickening of the same vessel wall (yellow arrow) (400x). D) A detail of the dermal/epidermal junction area, with some focal areas displaying spongiosis, edema (yellow arrow), lymphocytic infiltration predominantly perivascular lymphocytic infiltration (black arrow) and a subcorneal cleavage (blue arrow) (200x). E) Higher magnification (400x). F) A detail of the reticular dermis, with edema (400x). G) In few cases, we observed blister lumina containing erythrocytes but not containing inflammatory white blood cells or fibrin (black arrows) within a vertical column between two histologically adjacent acrosyringia. Stratum corneal keratin within these vertical columns is more transparent compared to adjacent corneal areas (yellow dots). H) Utilizing human epidermal extracts, we performed immunoblotting. We noted the presence of autoantibodies against 300, 250, 230, 190, 200, 160, 117, 97, 55, 45 and 34-kDa molecules in the El Bagre EPF sera (lanes 1 10). Lane 11 is for anti-desmoglein (Dsg1) monoclonal antibody (Progen, Heidelberg, Germany) reactive with the 160-kDa band. Lane 12 is for a negative normal control.

cell surface staining at a 1 : 40 titer. Anti-human fibrinogen antiserum displayed the strongest positivity in these cases, with titers of 1 : 160 in the central lamina propia; the pattern resembled a fine plumage at the BMZ. Collagen IV seemed to colocalize with the immunoreactivity to fibrinogen. When antiserum to IgM and albumin were used, BMZ reactivity was seen, but mostly in epidermal basal cells. IgE was also positive at a 1 : 40 titer, and associated with perinuclear staining around the keratinocytes. Both

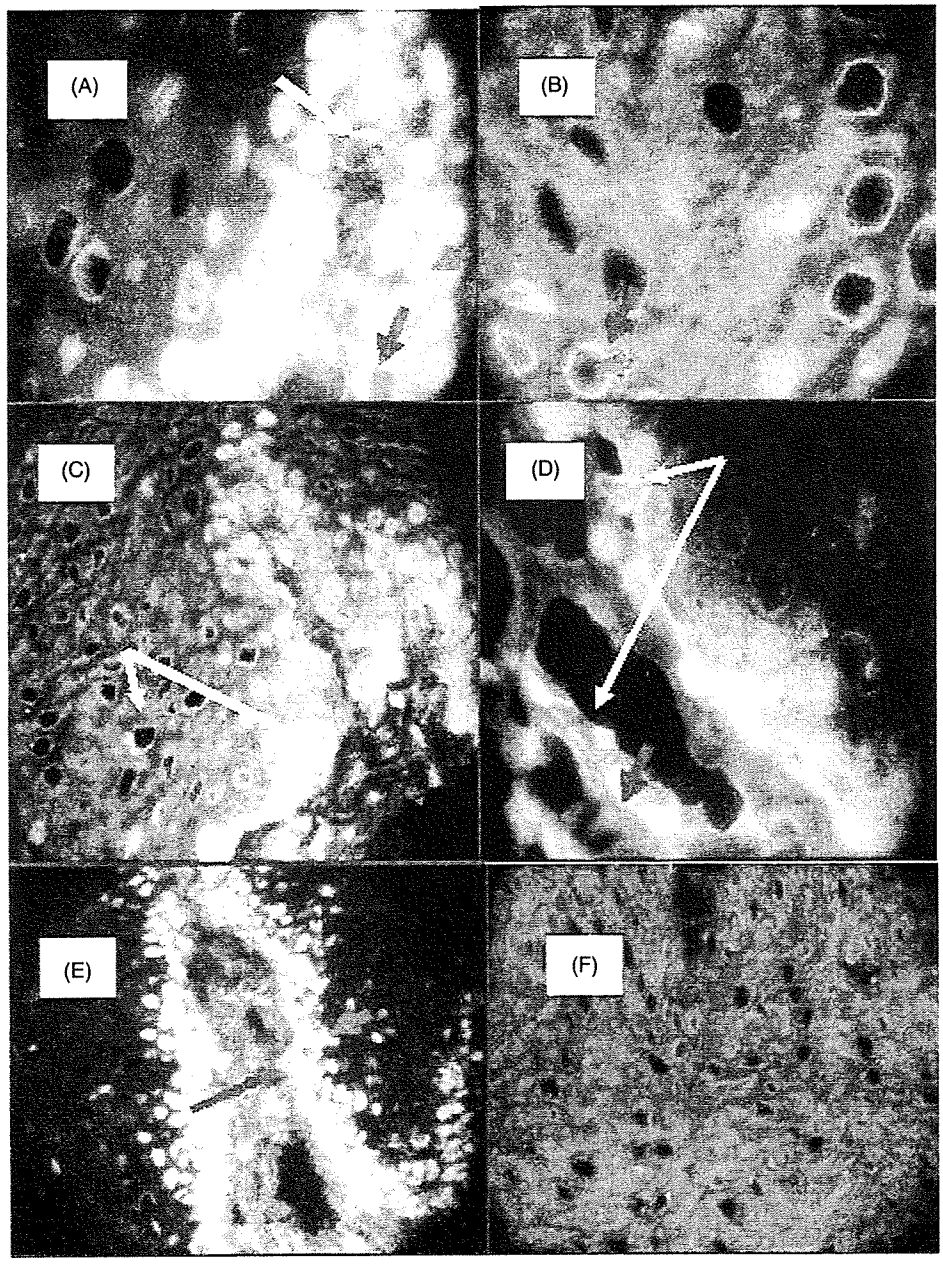

Fig. 3. Indirect immunofluorescence (IIF) for El Bagre endemic pemphigus foliaceus (EPF) patients utilizing monkey esophagus as a substrate. A) FITC conjugated anti-human fibrinogen antiserum showed reactivity to the basal membrane zone (BMZ) (green stain) (red arrow). This immunostaining colocalized with monoclonal antibodies to collagen IV (red stain) (white arrow). In addition, the nuclei of the keratinocytes were counterstained with Dapi (blue) (fuchsia arrow) (100x). B) The IIF also showed positive staining with FITC conjugated anti-human IgM antiserum, this staining was manifested as a granular intracytoplasmic staining within the keratinocytes, mostly in the basal membrane areas (green staining) (red arrow). Keratinocyte nuclei were also counterstained with Dapi in this procedure (blue stain) (fuchsia arrow) (400x). C) IIF for (B) was repeated; i.e. FITC conjugated anti-human IgM antiserum showed intracytoplasmic staining in the epithelial keratinocytes (red arrow) and at the BMZ. In this second procedure, colocalization with antibody to collagen IV was also observed (white arrows) (400x). D) Positive deposits of FITC conjugated anti-human IgG antiserum were noted at the BMZ (green stain) (red arrow). In this procedure, the antibody to collagen IV colocalized with the IgG antibody (orange stain) was observed (white arrow). The nuclei of the keratinocytes were again counterstained with Dapi (blue stain) (fuchsia arrow) (600x). F) FITC conjugated anti-human IgE antiserum, showing perinuclear reactivity within the keratinocytes (red arrow) (100x).

Dapi and collagen IV performed well in mapping and colocalization studies.

The IB results

Immunoreactivity to 160kDa Dsg1, in addition to plakins (envoplakin and periplakin), was detected in El Bagre EPF patients (Fig. 2H). Of interest, a band of about 200 kDa and several other bands were also noticed in the sera from the patients with the lupus-like histopathologic pattern that require further scrutiny.

Discussion

The problem in histopathologic differentiation of endemic pemphigus disease is the fact that the current scientific literature regarding FS was generated from the most prevalent clinical form of the disease. Through our 11 years of field work with this El Bagre- EPF, we were able to follow the patients for longer periods. We were thus able to observe and document temporal changes in the clinical disease, and show the wide range of histopathologic patterns detected in the patients. Our results show some differences with other types of superficial pemphigus. As in pemphigus erythematosus, the lesions do exhibit histologic features shared with other dermatoses.[18,19] The clinical, histopathologic and immunologic data correlate with the wide-ranging number of autoantibodies present in people affected by this variant of EPF. Autoantigens detected in this variant of El Bagre EPF include Dsgs, multiple plakin molecules and other antigens whose nature remains unknown.[10 16] Such a pattern of immunoreactivity may be reflected in the polymorphous clinical patterns detected in the patients affected by El Bagre EPF. One of the largest series of histopathologic studies in patients affected by FS was reported by Furtado in 1959.[4] The study consisted of 213 sections from 183 patients of FS. The most common histologic findings were (a) acantholysis (in 91.8% sections), (b) acanthosis (in 74.8%), (c) acantholytic cells (in 68.5%), (d) bulla formation (in 47%), (e) dyskeratosis (in 38.5%), (f) hyperkeratosis (in 31.9%), (g) epidermal appendage changes (in 31.9%), (h) papillomatosis (in 26.3 %) and (i) hyperpigmentation (in 25.5%).[4] Other findings such as chronic dermatitis, psoriasiform dermatitis and pustular dermatitis have also been described in patients with FS.[1,2,5] These specific findings are also found within our data. Larger comparative studies might yield insights by simultaneously correlating clinical, histologic and immunologic findings. Of interest was the presence of the melanophage-like cells in the skin of these patients; this finding seems to arise not solely because of a passive pigment transfer process. We base our suggestion on three findings: (a) the presence of patchy, liquefactive areas at the basement membrane zone detected by H&E staining; (b) focal immunoreactivity at the basement membrane zone identified by DIF and IIF and (c) the presence of multiple melanophages and dendritic-like cells in these areas. These cells probably display an active pigment scavenging function; they may provide a barrier against ionizing radiation, and also perform as scavengers of cytotoxic free radicals and intermediate molecules.

Of note is the fact that patients taking prednisone 20–40 mg/day when the biopsies were performed showed some of the atypical histologic features (i.e. different from the usual PF pattern). Those biopsies showed papillomatosis and hyperkeratosis and a large number of melanophages. Our observation is of importance, since such medication can in fact affect the pattern appreciated on histologic review.

Two important findings were the detection of inflammation in the palms and mild acantholysis in these biopsies. Also, frank separation at the granular layer was not seen in the palms, in contradistinction to non-glabrous skin areas; in the palms, cleavage was found in subcorneal areas. As the corneal layer is thickened in the palms, we cannot determine based on our current data whether this finding was actually part of the disease process in the palms or was due to an artefact in the biopsy fixation process. Of additional interest is the issue of whether sebaceous glands play an active role in the intensity of this disease on non-glabrous skin, since all the other epidermal appendages are present in the glabrous skin, with exception of these glands. Of importance is the fact that in the chronic stages of the disease, the presence of histologic scleredermoid changes, lipodermatosclerosis and periappendageal inflammation may cause the loss of appendages in patients affected by El Bagre EPF.

In conclusion, our findings indicate that the pathophysiology of El Bagre EPF represents a dynamic, largely undefined process, with possible overlapping features of other autoimmune molecular processes. We suggest that the diagnosis of EPF should not be based solely on histopathologic findings, but also be accompanied by clinical, epidemiologic and immunologic criteria.

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Circulating IgA and IgE autoantibodies in antilaminin-332 mucous membrane pemphigoid

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Conflicts of interest
None declared.

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Summary

Background Antilaminin-332 mucous membrane pemphigoid (MMP) is a chronic autoimmune bullous disease that is often associated with internal malignancy. IgG autoantibodies against laminin-332 in patients with MMP are well documented; however, IgA and IgE autoantibodies against laminin-332 have not yet been described.

Objectives To characterize IgA and IgE autoantibodies binding to laminin-332 in sera from patients with antilaminin-332 MMP.

Methods Sera and skin samples from four patients who met the following criteria were used: (i) subepidermal blistering lesions present on the mucous membranes; (ii) *in vivo* deposition of IgG along the epidermal basement membrane zone of sampled skin; (iii) circulating IgG antibasement membrane zone antibodies that react with the dermal side of salt-split normal human skin; and (iv) circulating IgG autoantibodies that do not show positivity against type VII collagen or 200-kDa protein (p200 antigen) in immunoblot analysis using dermal extracts. Circulating IgG/IgA/IgE class autoantibodies against laminin-332 were determined by immunoblotting.

Results Circulating IgG autoantibodies against the γ2, α3/γ2, α3 and α3/β3/γ2 subunits of laminin-332 were demonstrated in sera from four patients, respectively. Serum from one of the four patients showed IgA reactivity with the α3/β3/γ2 subunits of laminin-332. Serum from one of the four patients showed IgE reactivity with the γ2 subunit of laminin-332. The control sera failed to display IgG/IgA/IgE reactivity to laminin-332.

Conclusions In addition to IgG autoantibodies, circulating IgA and IgE autoantibodies against laminin-332 are detectable in a subset of patients with antilaminin-332 MMP.

Mucous membrane pemphigoid (MMP) is a heterogeneous group of autoimmune subepidermal blistering disorders that are characterized by circulating autoantibodies against epidermal basement membrane zone (BMZ) components and mucous membrane involvement.[1] To date, several epithelial components in the BMZ have been identified as autoantigens recognized by autoantibodies in patients with MMP. These include laminin-332 (α3, β3 and γ2 subunits), laminin-311 (α3 subunit), BP230 (BPAG1), type XVII collagen (COL17), type VII collagen (COL7) and the β4 integrin subunit.[1] Among these, laminin-332, previously called laminin-5 or epiligrin, is a major autoantigen in patients with MMP.[2–7]

Clinical manifestations of patients with antilaminin-332 MMP (L332-MMP) are severe and often include blistering and erosions of the conjunctivae, oral mucosa, laryngeal tract and oesophagus.[6] Recent studies showed that patients with L332-MMP have an increased relative risk of solid cancer.[8,9] IgG autoantibodies against laminin-332 in patients with MMP are well documented. In addition, the pathogenicity of IgG antibodies against laminin-332 has been clarified using *in vivo* mouse models.[10,11] In contrast to IgG, other immunoglobulin subtypes, such as IgA and IgE, have not been described as autoantibodies in patients with L332-MMP.

This study aims to characterize the immunoglobulin subtypes of circulating autoantibodies in sera from patients with L332-MMP. Our data demonstrate that IgA and IgE autoantibodies are present in a subset of patients with L332-MMP.

Materials and methods

Antibodies

Affinity-purified fluorescein isothiocyanate-conjugated goat antihuman IgG, horseradish peroxidase (HRP)-conjugated goat $F(ab')_2$ antimouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, U.S.A.), HRP-conjugated rabbit antihuman IgG, HRP-conjugated rabbit antihuman IgA (Dakocytomation, Glostrup, Denmark) and monoclonal mouse antihuman IgE (GE-1) (Sigma Aldrich, St Louis, MO, U.S.A.) were used in this study.

Immunofluorescence analysis

Direct immunofluorescence was performed on perilesional skin biopsy specimens from patients. Indirect immunofluorescence was performed on 1 mol L^{-1} NaCl-split normal human skin as described previously.[12]

Immunoblot analysis

Normal human dermal extracts were derived as described previously.[13] Briefly, fresh normal human skin was incubated in phosphate-buffered saline containing 2 mmol L^{-1} ethylenediaminetetraacetic acid and 1 mmol L^{-1} phenylmethylsulphonyl fluoride (PMSF) for 48 h at 4 °C. After dermal–epidermal separation, the dermis was extracted by treatment with urea-containing buffer (25 mmol L^{-1} Tris–HCl, pH 7·0, 8 mol L^{-1} urea and 1 mmol L^{-1} PMSF) for 2 h at room temperature. After centrifugation, supernatants were dialysed against distilled water for 48 h at 4 °C and lyophilized. Purified laminin-332 was a courtesy gift from Dr S. Amano, Shiseido Life Science Research Center, Yokohama, Japan.[14,15]

For immunoblotting of normal human dermal extracts and purified laminin-332, each sample was solubilized in Laemmli's sample buffer and applied on sodium dodecyl sulphate–polyacrylamide gels, and transferred on to nitrocellulose membrane. A Ponceau S stain was performed for total protein staining and visualized on a digital camera. The membrane was blocked for 1 h at room temperature in 3% skimmed milk in Tris-buffered saline. For IgG detection, blots were incubated with 1 : 20 diluted serum overnight at 4 °C. Bound antibodies were visualized enzymatically using 1 : 100 diluted HRP-conjugated rabbit antihuman IgG. For IgA detection, membranes were incubated with 1 : 20 diluted serum overnight at 37 °C, and then incubated in 1 : 50 diluted HRP-conjugated rabbit antihuman IgA for 3 h at room temperature. For IgE detection, membranes were incubated with 1 : 3 diluted serum overnight at 4 °C followed by 1 : 1000 diluted mouse antihuman IgE for 3 h at room temperature, and finally 1 : 500 diluted HRP-conjugated antimouse IgG for 3 h at room temperature. Colour was developed with 4-choro-1-naphthol in the presence of H_2O_2.

Patients

Sera and skin samples from four patients with L332-MMP were used in this study. These patients met the following criteria: (i) subepidermal blistering lesions present on mucosal surfaces; (ii) *in vivo* deposition of IgG along the BMZ in skin samples from patients; (iii) circulating IgG anti-BMZ antibodies that react with the dermal side of 1 mol L^{-1} NaCl-split skin; and (iv) circulating IgG autoantibodies that do not show positivity against type VII collagen or 200-kDa protein (p200 antigen) by immunoblot analysis using dermal extracts as described above. Direct and indirect immunofluorescence on perilesional skin samples and sera showed no IgA or IgE deposition at the BMZ for any of the four patients.

Case reports

Patient 1

A 77-year-old man with a 3-year history of rheumatoid arthritis noticed erosions on his oral mucosa 2 months before he was referred to our hospital. He had not taken any medication for his arthritis. Upon physical examination, multiple blisters and erosions were observed on his trunk, extremities and oral mucosa. Systemic corticosteroids gradually alleviated his skin and mucosal condition.

Patient 2

The patient was a 63-year-old man who had had rheumatoid arthritis for 5 years and was being treated with bucillamine. He noticed multiple bullae on his extremities and erosions on the oral mucosa and both conjunctivae 6 months before referral to our hospital. His symptoms showed no improvement at 2 months after discontinuation of the bucillamine. Physical examination revealed erosions on the oral mucosa and the whole body, and scarring on the conjunctivae. He refused further investigation and treatment.

Patient 3

A 62-year-old man with bronchial asthma and diabetes mellitus had complained of conjunctival congestion 5 years before referral. The diagnosis of ocular pemphigoid was made by ophthalmologists, and he was treated with systemic corticosteroids. He was referred to our hospital after his condition worsened with a tapering of the corticosteroids. Multiple bullae on his extremities, erosions on the oral mucosa and scarring of both conjunctivae were observed. Oesophageal involvement was noted. Cyclophosphamide in combination with prednisolone ameliorated his skin and mucosal condition, although the conjunctival scarring remained.

Patient 4

The patient was an 85-year-old man with end-stage carcinoma of the lung. Blisters and erosions appeared on his extremities,

trunk and oral mucosa. After systemic corticosteroid treatment was started, his skin symptoms improved.

Histopathology

Histopathological findings of perilesional skin samples from all patients revealed subepidermal blister formation with infiltration of inflammatory cells, including a few eosinophils. There were no notable differences in histopathological features between samples.

Results

IgG autoantibodies against purified laminin-332 in sera from the four patients

Ponceau S and control L332-MMP serum revealed four distinctive proteins that characterize laminin-332: 165-kDa processed α3 subunit, 145-kDa degraded α3 subunit, 140-kDa β3 subunit and 105-kDa γ2 subunit (Fig. 1a). Serum from patient 1 had circulating IgG autoantibodies against the γ2 subunit of laminin-332. Serum from patient 2 had circulating IgG autoantibodies against the α3 and γ2 subunits of laminin-332. Serum from patient 3 had circulating IgG autoantibodies against the α3 subunit of laminin-332. Serum from patient 4 had circulating IgG autoantibodies against all three subunits (α3, β3 and γ2) of laminin-332 (Fig. 1a).

IgA autoantibodies against purified laminin-332 were found in a subset of the patients with antilaminin-332 mucous membrane pemphigoid

Immunoblot analysis using purified laminin-332 showed that IgA autoantibodies from patient 3 showed reactivity against all three subunits (α3, β3 and γ2) (Fig. 1b).

Circulating IgE autoantibodies against purified laminin-332 were present in one of four patients

IgE autoantibodies from patient 1 tested positive for the γ2 subunit (Fig. 1c).

Healthy control sera failed to display any IgG/IgA/IgE reactivity to purified laminin-332 (Fig. 1a–c). Table 1 summarizes the four patients with L332-MMP, the immunoglobulin subtypes demonstrated to be autoantibodies and the antigenic subunits of laminin-332.

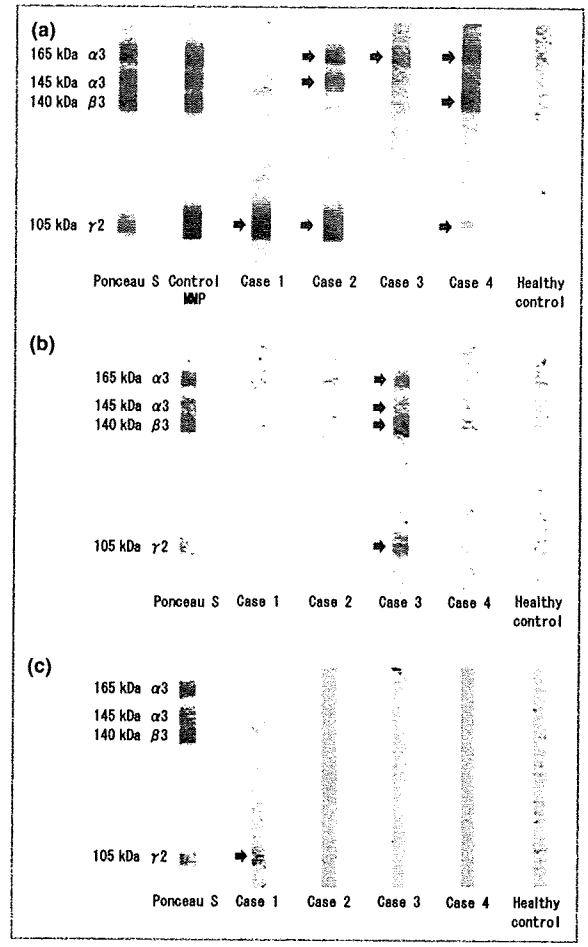

Fig 1. IgG, IgA and IgE autoantibodies against purified laminin-332. (a) Immunoblot analysis using purified laminin-332 revealed circulating IgG autoantibodies against the γ2 subunit, 105 kDa (arrow, case 1), the α3 and γ2 subunits, 165 kDa, 145 kDa and 105 kDa (arrows, case 2), the α3 subunit, 165 kDa (arrow, case 3), and all the α3/β3/γ2 subunits, 165 kDa, 140 kDa, 105 kDa (arrows, case 4) in sera from patients with mucous membrane pemphigoid. (b) IgA from case 3 serum reacted with all the α3/β3/γ2 subunits, 165 kDa, 145 kDa, 140 kDa, 105 kDa (arrows). (c) Case 1 serum had circulating IgE autoantibodies against the γ2 subunit, 105 kDa (arrow).

Discussion

IgG is the main immunoglobulin subtype that has been confirmed as an autoantibody against BMZ components in sera from patients with MMP. In sera from patients with L332-MMP, only IgG autoantibodies have been described so far. Previous studies revealed that passive transfer of rabbit antilaminin-332 IgG induces subepidermal blisters in neonatal mice.[10] Furthermore, antilaminin-332 IgG antibodies purified from human patients are known to induce subepidermal blistering in human skin grafts on SCID mice.[11] These *in vivo* experiments suggest that IgG antibodies against laminin-332 play a pathogenic role in MMP.

IgA autoantibodies are another major immunoglobulin subtype found in sera from patients with MMP, and these autoantibodies specifically recognize COL17 (anti-COL17 MMP).[16–21] Recent studies have revealed that passive transfer of monoclonal mouse IgA against the linear IgA dermatosis antigen, which is the shed ectodomain of COL17, into human skin grafts transplanted on SCID mice produces subepidermal separation and neutrophil infiltration.[22] This

Table 1 Summary of patients with antilaminin-332 mucous membrane pemphigoid, autoantibody immunoglobulin subtypes, and antigenic subunits of laminin-332

Patient	Sex/age (years)	Concurrent illness	Treatment	Autoantibody subclass			Antigenic subunits of laminin-332
				IgG	IgA	IgE	
1	M/77	Rheumatoid arthritis	PSL	+	–	+	γ2 (IgG), γ2 (IgE)
2	M/63	Rheumatoid arthritis	PSL	+	–	–	α3/γ2 (IgG)
3	M/62	Bronchial asthma, diabetes mellitus	PSL + CPM	+	+	–	α3 (IgG), α3/β3/γ2 (IgA)
4	M/85	Lung carcinoma	PSL	+	–	–	α3/β3/γ2 (IgG)

PSL, prednisolone; CPM, cyclophosphamide.

supports the theory that IgA autoantibodies also play a pathogenic role in IgA-related autoimmune bullous diseases. It was recently argued that IgE autoantibodies play a pathogenic role in autoimmune blistering diseases. Some patients with bullous pemphigoid (BP) have IgE autoantibodies against COL17[18,23–26] and BP230,[23,26,27] and injection of purified IgE against COL17 produced subepidermal blistering of normal human skin grafts in immunodeficient mice.[28,29] Therefore, IgE might also play an important role in the pathogenesis of certain autoimmune blistering diseases. However, IgA and IgE autoantibodies against laminin-332 in MMP sera have not been described.

The correlation between clinical manifestations and the immunoglobulin subtypes in autoantibodies is difficult to define. This is because of the limited number of patients included in our study, although patient 3 in this study, with IgA autoantibodies against laminin-332, had severe conjunctival involvement. Previous studies showed IgE autoantibodies in cases of severe BP.[23,24] In our study, patient 1, with IgE autoantibodies against laminin-332, showed a good response to systemic corticosteroid treatment without sequelae.

The concentration of IgA/IgE is much lower than that of IgG, which may explain the difficulty of detecting circulating IgA/IgE antibodies. Immunofluorescence analysis of the patients with MMP in our study showed no detectable deposition of IgA or IgE at the BMZ, although IgE and IgA autoantibodies against laminin-332 were detected by immunoblot in patients 1 and 3, respectively. In previous studies, immunoblot analysis also detected anti-COL17 IgA or IgE autoantibodies in sera from patients whose skin specimens and sera showed no deposition of IgA or IgE at the BMZ.[18] This phenomenon can be explained by the difference in sensitivity between immunofluorescence and immunoblot.

IgG is still the main immunoglobulin subtype of autoantibodies against laminin-332. Nevertheless, IgA and IgE autoantibodies against laminin-332 were detectable in a small subset of patients with MMP. In summary, this study is the first report to describe IgA and IgE autoantibodies against laminin-332 in patients with MMP. Further study is needed to elucidate the frequency and pathogenicity of IgA/IgE antibodies in patients with L332-MMP.

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A case of antilaminin 332 mucous membrane pemphigoid showing a blister on the bulbar conjunctiva and a unique epitope on the α3 subunit

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MADAM, A 57-year-old Japanese woman developed severe erosive lesions on the oral mucosae including buccal mucosae, gingivae, hard palate and soft palate, as well as bleeding and crust formation on the nasal mucosae in August 2007. She also showed bloody blisters and crusted erosions 2–4 cm in size scattered on the trunk and extremities. Oral minocycline 200 mg daily was given. However, the erosive lesions on the oral and nasal mucosae continued to develop and a clear blister 5 × 8 mm in size appeared on the left bulbar conjunctiva (Fig. 1). Therefore, minocycline was replaced by oral prednisolone 40 mg daily, but the mucosal and skin lesions still continued. Then, a combination therapy of oral prednisolone 40 mg daily and dapsone 75 mg daily was initiated. The oral and nasal mucosal lesions healed without any scarring. The blister on the bulbar conjunctiva disappeared without any scarring or sight disturbance. The skin lesions also healed leaving slight scarring. Then, the dose of prednisolone was tapered gradually without lowering the dose of dapsone, but no mucosal or skin lesions recurred. In March 2008, the patient was free from any mucosal or skin lesions on a combination therapy of prednisolone 5 mg daily and dapsone 75 mg daily.

Histopathology of a skin biopsy specimen from the back showed a subepidermal bulla with massive neutrophil infiltration and scattered eosinophils. Direct immunofluorescence did not show positive results, probably because of damage to the basement membrane zone of the biopsy specimen. Indirect immunofluorescence using normal human skin detected IgG, but not IgA, antibasement membrane zone antibodies at a titre of 1 : 40, which reacted exclusively with the dermal side of 1 mol L^{-1} NaCl-split skin. Immunoblot analysis using purified human laminin 332 as a substrate was performed as described previously.[1,2] In this study, IgG antibodies of a representative control patient with antilaminin 332 mucous membrane pemphigoid (MMP) reacted with both the 165-kDa form and the 145-kDa form of the α3 subunit, the 140-kDa β3 subunit and the 105-kDa γ2 subunit of laminin 332 (Fig. 2, lane 1). IgG antibodies of the present case reacted clearly and exclusively with the 165-kDa form of the α3 subunit (Fig. 2, lane 2). From these results, the diagnosis of antilaminin 332 MMP was confirmed in this patient.

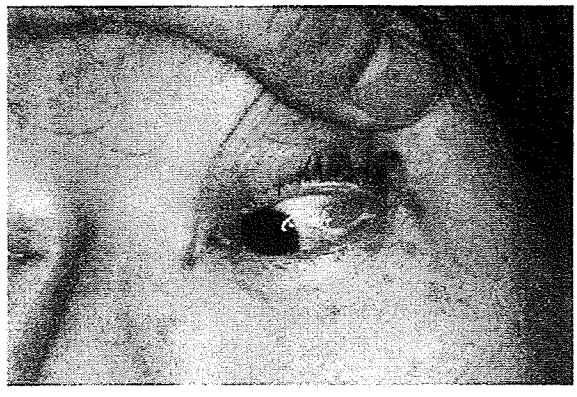

Fig 1. Clinical features of the ocular mucosa. A clear blister was seen on the bulbar conjunctiva of the left eye.

Cicatricial pemphigoid shows blisters and erosive lesions mainly on the mucous membranes, such as the oral, ocular, nasal, laryngeal, pharyngeal and genital mucosae, and skin lesions appear occasionally. These lesions heal with scar formation. However, because this subset of autoimmune bullous disease mainly shows mucosal lesions, and oral mucosal lesions usually heal without scarring, the term 'MMP' is now commonly used, following a consensus meeting.[3] The members of the consensus meeting agreed that dapsone or tetracycline (or minocycline) may be effective when the lesions are localized to the oral mucosae. However, stronger therapies have to be selected when progressive lesions are seen on the ocular or laryngeal mucosae, which may lead to blindness or dyspnoea, respectively.

MMP is highly heterogeneous, but there are at least three major subtypes: anti-BP180 MMP, antilaminin 332 MMP and ocular MMP. About 80% of cases of MMP are anti-BP180

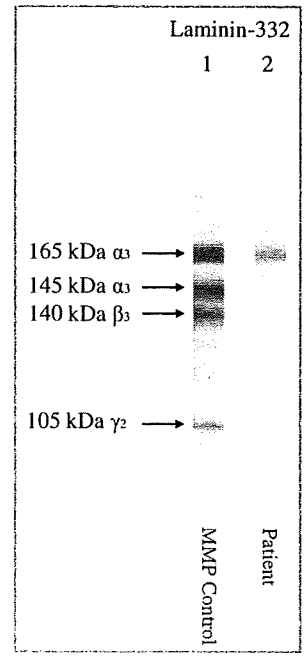

Fig 2. The result of immunoblot analysis using purified human laminin 332. A control antilaminin 332 mucus membrane pemphigoid (MMP) serum reacted with all the subunits of laminin 332, including the 165-kDa and 145-kDa forms of the α3 subunit (lane 1). The serum of the present case reacted clearly and exclusively with the 165-kDa form of the α3 subunit (lane 2). The position of each subunit is shown on the left.

MMP, which shows IgG and IgA antibodies to the carboxyl-terminus of BP180.[4] A further 10–20% of cases of MMP are antilaminin 332 MMP, which shows IgG antibodies to laminin 332 (previously called epiligrin or laminin 5).[5,6] Ocular MMP shows exclusive ocular mucosal lesions, although the autoantigen for this group has not been clearly identified.

Although ocular lesions are commonly seen in MMP, they are usually hyperaemia or erosions which result in symblepharon or epithelialization over the cornea. A clear blister on the bulbar conjunctiva is rarely seen. In our case, a clear solitary blister appeared on the bulbar conjunctiva of the left eye without apparent hyperaemia or erosion. This blister quickly disappeared without any scar formation after the treatment of prednisolone and dapsone was initiated. In addition, a unique clinical feature of our case was the excellent effectiveness of dapsone on all the mucosal and skin lesions.

Another interesting result for this case was the unique reactivity in immunoblot analysis using purified laminin 332. We have shown that the IgG antibodies in patients with antilaminin 332 MMP react with the three subunits of laminin 332, i.e. the α3 subunit, β3 subunit and γ2 subunit, in various patterns.[2] In particular, most patient sera react with both the 165-kDa and 145-kDa forms of the processed α3 subunit, but not with the 200-kDa unprocessed α3 subunit. In our preparation of laminin 332, the 200-kDa unprocessed α3 subunit is not present. The 145-kDa protein is considered to be a degradation product from the 165-kDa processed α3 subunit, although it is not known where the digested 20-kDa fragment resides in the 165-kDa processed α3 subunit. In the previous study,[2] most patient sera reacted with both the 165-kDa and 145-kDa proteins, indicating that the epitopes for these sera are present on the domain common for both proteins. In contrast, the serum of the present case reacted only with the 165-kDa protein, but not with the 145-kDa protein, indicating that the epitope for this serum is present on the 20-kDa fragment which is digested by some protease. Therefore, it is worthwhile identifying the position of the 20-kDa fragment within the 165-kDa form of the processed α3 subunit. From the results of our previous reports,[1,7] the 20-kDa fragment is assumed to correspond to the domain IIIa region of the 165-kDa form of the processed α3 subunit. A study using recombinant proteins of the α3 subunit is now going on to confirm this speculation.

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Key words: *bulbar conjunctiva, epitope, laminin 332, mucous membrane pemphigoid*

Conflicts of interest: none declared.

Fine mapping of the human *AR/EDA2R* locus in androgenetic alopecia

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MADAM, Male-pattern baldness (androgenetic alopecia, AGA) is the most common form of hair loss among humans and affects up to 80% of men by the age of 80 years.[1] Hamilton was the first to describe the two essential aetiological factors underlying the development of AGA: genetic predisposition and hormone dependency.[1] Several studies[2–8] have reported that the X-chromosomal locus containing the genes for the androgen receptor (*AR*) and the ectodysplasin A2 receptor (*EDA2R*) is the major genetic susceptibility locus for AGA. However, the association signals in these studies have been inconsistent, and the causative variant or gene has not yet been unequivocally identified. In the present study, we aimed to resolve these inconsistent data through systematic fine mapping of the *AR/EDA2R* locus in the largest sample of patients with AGA investigated to date.

We defined the associated *AR/EDA2R* locus as the region in which single-nucleotide polymorphisms (SNPs) had P-values of < 0·01, on the basis of the findings of our previous genome-wide association study (GWAS).[7] To maximize the efficiency of the study, we used the tagger algorithm of the Haploview software (http://www.broadinstitute.org/mpg/

The findings reported by Jones *et al.* are of importance when considering the optimal therapeutic strategy to initiate in a given patient with CTCL. Because methylation of the Fas promoter is not observed in all patients with SzS, the use of hypomethylating drugs may not be equally effective in restoring sensitivity to Fas-mediated apoptosis in all patients. Thus, not only is the existence of potent hypomethylating agents important, but their use will require a "personalized" medical approach in which these agents are employed in patients having tumors with positional methylation of the Fas CpG island.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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See related article on pg 1040

How Does Intramolecular Epitope Spreading Occur in BPAG2 (BP180)?

Takashi Hashimoto[1], Takahiro Hamada[1], Teruki Dainichi[1], Norito Ishii[1], Tadashi Karashima[1], Takekuni Nakama[1] and Shinichiro Yasumoto[1]

Several studies have suggested that autoantibodies directed against multiple epitopes occur via epitope spreading in autoimmune bullous skin diseases. However, the precise sequence of events in epitope spreading has not been elucidated for any of the epidermal autoantigens. In this issue, using a transgenic mouse model, Di Zenzo *et al.* report that intramolecular epitope spreading does occur for human BPAG2.

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In order to investigate the mechanism of epitope spreading for BPAG2 (BP180 or type XVII collagen), Di Zenzo *et al.* (2010, this issue) performed a sophisticated set of experiments using transgenic mice harboring human BPAG2. To immunize mice with human BPAG2, skin samples from transgenic mice that expressed human BPAG2 were grafted onto syngeneic mice. Sequential serum samples were then obtained from the immunized mice, and antibodies against human BPAG2 were detected by an enzyme-linked immunosorbent assay using recombinant proteins for four intracellular domains (ICDs) and three extracellular domains (ECDs) of human BPAG2. Most grafted mice

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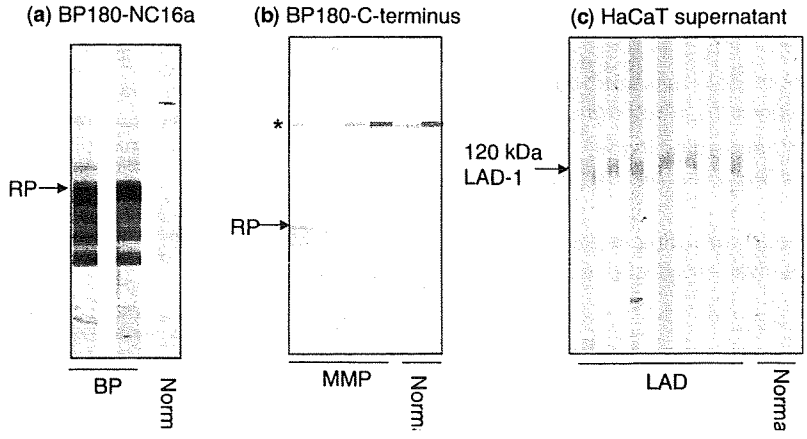

Figure 1. Representative results of immunoblot analyses for BPAG2 (BP180) using three different antigen sources. (**a**) Bacterial recombinant protein of the NC16a domain of BPAG2. IgG antibodies in bullous pemphigoid (BP) sera reacted with this recombinant protein (RP). (**b**) Bacterial recombinant protein of C-terminal domain of BPAG2. IgG antibodies in anti-BP180-type mucous membrane pemphigoid (MMP) sera reacted with this RP. The upper protein band marked with an asterisk indicates nonspecific reactivity because it is also shown by normal controls. (**c**) Concentrated supernatant sample from cultured HaCaT cells. IgA antibodies in lamina lucida type of linear IgA bullous dermatosis (LAD) sera reacted with the 120-kDa LAD-1 antigen.

initially developed anti-BPAG2 antibodies directed against ECD epitopes. Subsequently, some of the mice developed antibodies to additional ECD epitopes and to ICD epitopes. In general, the titers of antibodies against the ECD epitopes were high, whereas antibodies against the ICD epitopes were low, and they were detectable for shorter periods of time. An interesting observation was that the development of antibodies against ICD epitopes correlated with graft loss, but rejection occurred by an unknown mechanism. Thus, Di Zenzo *et al.* confirmed successfully and directly that epitope spreading does occur in this animal model of an autoimmune bullous skin disease.

Multiple epitopes occur in the autoantigens that characterize autoimmune bullous diseases

Epitope spreading has been shown to occur in several autoimmune bullous skin diseases (Chan *et al.*, 1998). The multiple epitopes on desmoglein 1 (Dsg1) or Dsg3 are targets of antibodies found in sera from patients with pemphigus vulgaris, pemphigus foliaceus, and paraneoplastic pemphigus as determined by ELISA assays using domain-swapped molecules between human Dsg1 and Dsg3 (Futei *et al.*, 2003). Recently, these results were confirmed in an ELISA assay using newly elaborated domain-swapped molecules of human Dsg1 and Dsg3 against the human Dsg2 backbone (Chan *et al.*, in press; B Ohyama *et al.*, personal communication). Previously, we demonstrated that paraneoplastic pemphigus sera had autoantibodies against multiple epitopes in human envoplakin and periplakin, two major autoantigens found in paraneoplastic pemphigus, as demonstrated by an ELISA assay, using bacterial recombinant proteins from various domains of envoplakin and periplakin (Nagata *et al.*, 2001). We showed that bullous pemphigoid sera had autoantibodies against multiple epitopes in the various domains of human BPAG1 (BP230), particularly to the C-terminal globular domain, by immunoblot analysis using bacterial recombinant proteins of various domains of human BPAG1 (Hamada *et al.*, 2001). We also showed that anti-basement membrane zone antibodies in the sera of patients with epidermolysis bullosa acquisita reacted with distinct epitopes in the NC1 domain, the central collagenous domain, and the NC2 domain by immunoblot analysis using bacterial recombinant proteins from selected domains of human type VII collagen and immunoelectron microscopy (Ishii *et al.*, 2004).

These studies strongly suggest that intramolecular epitope spreading occurs in several autoimmune bullous skin diseases. Although the previous studies detected autoantibodies against multiple epitopes, no study detailed the sequential development of autoantibodies to different epitopes over time. Di Zenzo *et al.* (2010) have now shown that epitope spreading actually takes place. They found that mice immunized against human BPAG2 occasionally developed autoantibodies against some epitopes present in the ICD of BPAG2, although such autoantibodies appeared late and were less persistent. Interestingly, we found that pemphigus patients sometimes have autoantibodies that react against the ICD of Dsg1 and Dsg3 (Ohata *et al.*, 2001). Thus, the study by Di Zenzo *et al.* also confirms that antibodies against the ICD of transmembranous antigens can occur in autoimmune bullous skin diseases, probably also by epitope-spreading mechanisms. The mechanism by which such antibodies develop remains unknown.

BPAG2 is the most suitable autoantigen for studying mechanisms of epitope spreading

Autoantibodies to distinct epitopes within BPAG2 develop in a variety of autoimmune subepidermal bullous skin diseases. First, anti-basement membrane zone autoantibodies in both bullous pemphigoid and herpes gestationis were reported to preferentially react with the NC16a domain of BPAG2 (Matsumura *et al.*, 1996). In addition, IgG and IgA antibodies in anti-BP180 type mucous membrane pemphigoid have been shown to react with the C-terminal domain of BPAG2 (Nie and Hashimoto, 1999). Furthermore, we showed that IgA antibodies in lamina lucida–type linear IgA bullous dermatosis reacted with epitope(s) within the fifteenth collagenous domain of BPAG2, which is hidden in the intact 180-kDa BPAG2 molecule (Nie *et al.*, 2000). For these reasons, BPAG2 is considered the most suitable antigen with which to elucidate mechanisms of epitope spreading in autoimmune bullous skin diseases. Figure 1 shows representative immunoblot analyses for three antigen sources used routinely in our laboratory.

Clinical Implications

- Epitope spreading is the sequential development of new antibodies against seemingly less accessible regions of target proteins in autoimmunity.
- The identification of mechanisms of epitope spreading in the immunobullous diseases may lead to novel therapies that limit the process of spreading.
- Because of accessibility, the analysis of epitope spreading in skin disease may provide insight into pathogenic mechanisms in systemic autoimmune diseases and transplantation immunity.

Perspectives

Although Di Zenzo *et al.* (2010) demonstrated convincingly that intramolecular epitope spreading occurs in BPAG2, many questions remain. The first is why patients with bullous pemphigoid preferentially develop IgG autoantibodies to epitopes on the NC16a domain of BPAG2. Second, why do autoantibodies in bullous pemphigoid react with epitopes in the NC16a domain of BPAG2, whereas autoantibodies in anti-BP180–type mucous membrane pemphigoid react with epitopes in the C-terminal domain? More important, how do the antibodies directed against these distinct domains of BPAG2 result in different clinical features (i.e., large, tense skin blisters in bullous pemphigoid and predominant erosive mucosal lesions in anti-BP180-type mucous membrane pemphigoid)? Why do IgA antibodies in lamina lucida–type linear IgA bullous dermatosis react with specific epitopes in 120- and 97-kDa linear IgA bullous dermatosis (LAD)-1 antigens produced from 180-kDa intact BPAG2 by proteolytic processing (Nie *et al.*, 2000)? Future studies should unravel the mechanisms by which the hidden epitope in intact 180-kDa molecule (intact BPAG2) is exposed in linear IgA bullous dermatosis to autoantibodies against the 120- and 97-kDa LAD-1 antigens.

Finally, and perhaps most important, we do not know yet why the development of antibodies against ICD epitopes in human BPAG2 correlated with skin-graft loss. The relevance of this phenomenon to autoimmune bullous diseases remains to be determined.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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More or Less: Copy Number Alterations in Mycosis Fungoides

William M. Lin[1] and Michael Girardi[1]

Mycosis fungoides (MF) is the most common form of cutaneous T-cell lymphoma (CTCL), a heterogeneous group of non-Hodgkin's lymphomas of skin-homing T cells. MF may vary from limited patchy skin disease to extensive cutaneous plaque and tumor involvement to extracutaneous compartments of blood, lymph nodes, and viscera. Advances in genomic technologies have enabled the increasing characterization of genetic alterations in this malignancy; using this technology, investigators hope to understand MF's variable behavior and pathogenesis. In this issue, Salgado *et al.* identify regions of genomic DNA alterations from 41 MF samples and report associations with prognosis.

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In recognition that cancer is fundamentally dependent on genetic alterations (Vogelstein and Kinzler, 2004), the number of genomic

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