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Subclinical oral involvement in patients with endemic pemphigus foliaceus

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All authors have contributed significantly to this publication.

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ABSTRACT

Background: We have described a variant of endemic pemphigus foliaceus (EPF) in El Bagre area known as pemphigus Abreu-Manu. Our previous study suggested that Colombian EPF seemed to react with various plakin family proteins, such as desmoplakins, envoplakin, periplakin BP230, MYZAP, ARVCF, p0071 as well as desmoglein 1.

Objectives: To explore whether patients affected by a new variant of endemic pemphigus foliaceus (El Bagre-EPF) demonstrated oral involvement.

Materials and Methods: A case-control study was done by searching for oral changes in 45 patients affected by El Bagre-EPF, as well as 45 epidemiologically matched controls from the endemic area matched by demographics, oral hygiene habits, comorbidities, smoking habits, place of residence, age, sex, and work activity. Oral biopsies were taken and evaluated via hematoxylin and eosin staining, direct immunofluorescence, indirect immunofluorescence, confocal microscopy, and immunohistochemistry.

Results: Radicular pieces and loss of teeth were seen in in 43 of the 45 El Bagre-EPF patients and 20 of the 45 controls ($P < 0.001$) (confidence interval [CI] 98%). Hematoxylin and eosin staining showed 23 of 45 El Bagre-EPF patients had corneal/subcorneal blistering and lymphohistiocytic infiltrates under the basement membrane zone and around the salivary glands, the periodontal ligament, and the neurovascular bundles in all cell junction structures in the oral cavity; these findings were not seen in the controls ($P < 0.001$) (CI 98%). The direct immunofluorescence, indirect immunofluorescence, confocal microscopy, and microarray staining displayed autoantibodies to the salivary glands, including their serous acini and the excretory duct cell junctions, the periodontal ligament, the neurovascular bundles and their cell junctions, striated muscle and their cell junctions, neuroreceptors, and connective tissue cell junctions. The autoantibodies were polyclonal. IgA autoantibodies were found in neuroreceptors in the glands and were positive in 41 of 45 patients and 3 of 45 controls.

ABSTRACT **Conclusions:** Patients affected by El Bagre-EPF have some oral anomalies and an immune response, primarily to cell junctions. The intrinsic oral mucosal immune system, including IgA and secretory IgA, play an important role in this autoimmunity. Our data contradict the hypothesis that pemphigus foliaceus does not affect the oral mucosa due to the desmoglein 1-desmoglein 3 compensation.

Introduction

We have described a new variant of endemic pemphigus foliaceus in El Bagre, Colombia, South America (El Bagre-EPF, or pemphigus Abreu-Manu) [1-5]. El Bagre-EPF differs from other types of EPF clinically, epidemiologically, and immunologically. Previous studies have shown that patients affected by EPF in Brazil have some oral findings [7, 8]. Selected authors have described the presence of autoantibodies using hematoxylin and eosin (H&E) staining, direct and indirect immunofluorescence (DIF, IIF), and electron microscopy studies [9-11]. In the current study, our aim was to search for oral clinical lesions and an oral autoimmune response in patients affected by EPF in El Bagre, Colombia (El Bagre-EPF) [1-5] and to compare our findings with those described in the medical literature for Brazilian EPF patients.

Materials and Methods

Statement on Ethics

A human quality assurance review board approved the studies at the Hospital Nuestra Señora del Carmen in El Bagre, and all participants provided signed consent. The studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as established in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. We tested 45 patients affected by El Bagre-EPF and 45 controls from the endemic area matched by age, sex, demographics, comorbidities, work activities, weight, exposure to chemicals, socioeconomic status and income, and food intake. Thirty controls from the endemic area were healthy individuals. The other controls included patients with psoriasis, scleroderma, and chronic drug eruptions. All of the tests were performed in both cases and controls. The patients and controls were evaluated by H&E histology, DIF, IIF, confocal microscopy, immunoblotting, immunoprecipitation, and enzyme-linked immunosorbent assay. Only patients meeting diagnostic criteria for El Bagre-EPF were included; specifically, they had to display clinical and epidemiological features described for this disease, live in the endemic area [1,2], and have serum displaying intercellular staining (ICS) between epidermal keratinocytes and the basement membrane zone (BMZ) of the skin via either DIF or IIF using fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies to human total IgG or IgG4, as described elsewhere [1-5]. Furthermore, each patient had to be positive by immunoblotting for reactivity against Dsg1 [2, 3], as well as for plakin molecules; each patient's serum immunoprecipitated a concanavalin A affinity-purified bovine tryptic 45 kDa fragment of Dsg1 [4]; and each patient's serum had to yield a positive result using an enzyme-linked immunosorbent assay test when screening for autoantibodies to pemphigus foliaceus antigens [5].

Oral mucosa from the buccal mucosa was biopsied; 2 biopsies were taken, 1 for H&E staining and immunohistochemistry (IHC) and 1 for DIF. The skin was tested as previously described [1-5].

DIF, IIF, and IHC

We performed DIF and IIF as previously described [2, 3]. All samples were run with positive and negative controls. Several years ago, the first [12] discovered new autoantigens to several organs other than the skin. Because of the complexity of the immune response in these patients, we contacted other experts, including Dr. E. H. Beutner in the USA, Dr. Takashi Hashimoto in Japan, and Dr. W. W. Franke (formerly a professor at the University of Heidelberg in Germany). All agreed that our data indicated new autoantigens. We sent identical serum for study to these scientists, and all agreed this disorder was unique. A few months later, the primary owner of Progen Biotechnik (Heidelberg, Germany), Dr. W. W. Franke, commercialized these antibodies. Thus, we used the following antibodies from Progen: anti-ARCVF (Armadillo repeat gene deleted in velocardiofacial syndrome; cat. no. GP155), anti-desmoplakin (DP) 1 and DP2 (cat. no. 65146), anti-p0071 (cat. no. 651166), and anti-MYZAP (myocardium-enriched zonula occludens-1-associated protein; cat. no. 651169). Secondary antibodies were obtained from Thermo Fisher Scientific (Waltham, MA, USA), for ARCVF we used Alexa Fluor 555 goat anti-guinea pig, while for DP1, DP2, p0071, and MYZAP we used goat anti-mouse Texas red-conjugated IgG. We also used rabbit anti-junctional adhesion molecule 1 (JAM-A) (Thermo Fisher Scientific), as this antibody is positive against gap junction. We classified our findings as negative (-), weakly positive (+), moderately positive (++), and strongly positive (+++). For IHC, we utilized antibodies for α-1-antitrypsin, human matrix metalloproteinase 9 (MMP9), human tissue inhibitor of metalloproteinases 1 (TIMP-1), metallothionein,

and urokinase type plasminogen activator (all from Dako; Agilent Technologies, Santa Clara, CA).

Questionnaires on Oral Habits

Deleterious oral habits include bruxism parasomnias, traumatic brain injury, neurological disabilities, nail biting, morphological factors, temporomandibular joint dysfunction, tongue thrusting, mouth breathing, smoking habits, and chewing on plants and/or gum. Other questions included how often toothbrushes were changed, use of dental floss, dental visits, and frequency of brushing teeth.

Imgenex Microarray IIF Using Frozen Normal Oral Organs

Our microarray work was performed as described for our IIF; as our antigen source, we used a commercial human tissue microarray in duplicate from Imgenex Corporation (San Diego, CA, USA).

Confocal Microscopy

Confocal microscopy was performed as previously described [5,6].

Statistical Analysis

We used the Fisher exact test to compare 2 nominal variables (eg, positive and negative) of the antibody response. $P < 0.01$ with a 98% degree of confidence or more was considered statistically significant. We used the software GraphPad QuickCalcs (GraphPad Software Inc., La Jolla, CA, USA).

Results

Questionnaires on Oral Habits

Deleterious oral habits did not show any statistical significance between the cases and controls. The oral health habits were poor in all study participants (37/45 patients and 38/45 controls). Most never visited the dentist for economic reasons (43/45 patients and 42/45 controls), brushed their teeth at most once or twice a week (32/45 patients and 33/45 controls), and rarely used dental floss (40/45 patients and 41/45 controls). Overall, 42 of 45 El Bagre-EPF patients were taking oral prednisone in doses ranging from 5 to 40 mg/day. In addition, 3 of 45 controls were taking prednisone for systemic sclerosis or for psoriasis ($P < 0.001$) (confidence interval [CI] 98%).

Oral Evaluation

The most significant alteration in the El Bagre-EPF patients was the finding of multiple radicular pieces and loss of teeth in 43 of 45 El Bagre-EPF patients and in 17 of 45 controls ($P < 0.001$) (CI 98%) (Figure 1a). Furthermore, 14 of 47 El Bagre-EPF patients had no teeth (Figure 1). Leukoedema was found in 6 of 45 El Bagre-EPF patients and in no controls. Large varicosities were found in 10 of 45 patients at the base of the involved lingual renine veins or in vessels of the ventral surface of the tongue or the floor of the mouth, with no control varicosities recorded. Small ulcers were seen in the palatal mucosa in 5 of 45 El Bagre-EPF patients. Dental caries were also found in most participants.

H&E Staining

The H&E staining showed that 23 of 45 El Bagre-EPF patients had corneal and/or subcorneal epidermal blisters and dermal edema and lymphohistiocytic infiltrates under the BMZ and around the salivary glands (including their serous acini and the excretory duct cell junctions and the neurovascular bundles). These findings were not observed in the controls ($P < 0.001$) (CI 98%) (Figure 1b).

DIF, Confocal Microscopy, and Imgenex Microarray Studies

In Table 1, we present the results of our autoantibody findings in the skin and the oral mucosa, including their strength and colocalization with commercial antibodies to ARVCF, MYZAP, DP I-II, p0071, and JAM-A. In both anatomic areas, autoantibodies were polyclonal in nature with a prevalence of IgG and fibrinogen in the acute cases; in chronic cases (>2 years of disease), IgM was most commonly seen ($P < 0.001$) (CI 98%) (Figure 1). The controls were uniformly negative. The El Bagre-EPF patients' periodontal ligaments have polyclonal autoantibodies on 43 of 45 compared to 0 of 45 controls. Multiple structures in the oral mucosa displayed their strongest autoreactivity with IgA compared with their anatomic correlates in the skin ($P < 0.001$) (CI 98%) (see Table 1). The neuroreceptors in the salivary glands were very positive (+++) in most El Bagre-EPF patients compared with the controls ($P < 0.001$) (CI 98%). The controls demonstrated secretory IgA in the salivary glands, including serous acini and the excretory duct cell junctions; these findings were also noted in the El Bagre-EPF patients ($P < 0.001$) (CI 98%) (Figures 1 and 2). The El Bagre-EPF autoantibodies colocalized 100% with the commercial antibodies to ARVCF, p0071, DP I-II, MYZAP, and ARVCF ($P < 0.001$) (CI 98%). Albumin autoantibodies also colocalized with JAM-A used as control. When using antibodies to IgG we observed neutrophil extracellular traps coming from the dermal vessels. In the BMZs of the salivary glands (including in serous acini and excretory duct cell junctions), IgG was positive to some unique cells that may be stem cells. Unique individual cells resembling lymphocytes were positive with C3c, C1q, IgE, and IgG in an opsonized manner. Several cell junctions were positive in the epidermis but did not show the classic fishnet-like intercellular stain between keratinocytes commonly seen in pemphigus. Rather the staining was dot-like and on cell junctions.

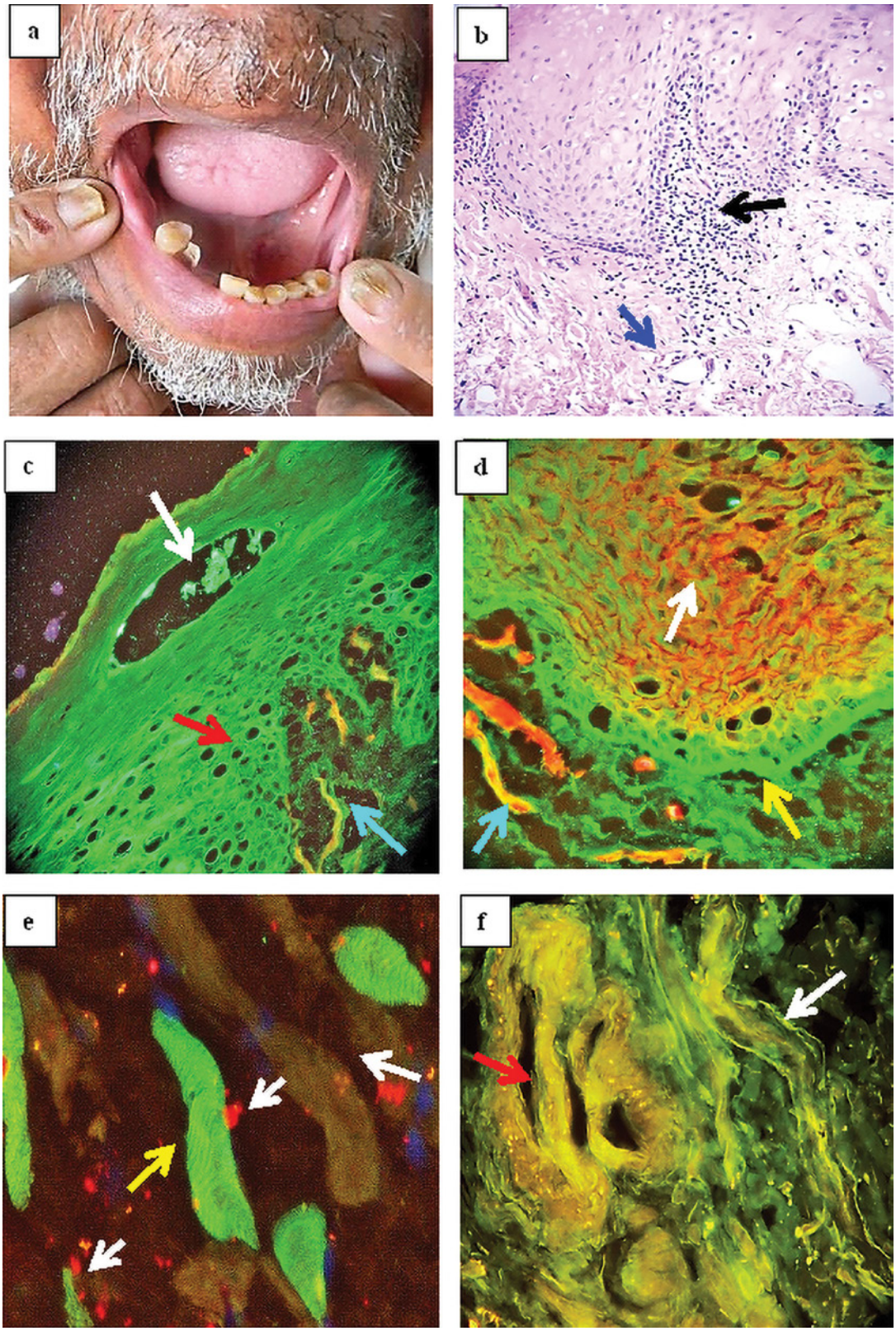

Figure 1. (a) Missing teeth in one El Bagre–EPF patient. (b) H&E staining of the oral mucosa, showing edema in the mucosa; in the dermis, a lymphohistiocytic infiltrate (black arrow) and dilation of a blood vessel (blue arrow) (200×). (c) DIF showing positive staining with FITC conjugated anti-fibrinogen antibodies in intracorneal blister (light green staining; white arrow) and pericytoplasmic staining of the epidermal keratinocytes (light green staining; red arrow) (200×) and stain in the vessels (yellow-green staining; light blue arrow). (d) DIF showing positive staining with FITC conjugated IgG antibodies against the BMZ (green staining; yellow arrow), as well as against upper dermal blood vessels with ULEX (yellow staining, resulting from the colocalization of FITC [green] and Texas red [red]; light blue arrow) (200×). (e) DIF showing positive staining with FITC conjugated fibrinogen antibodies (green staining), colocalizing with MYZAP Alexa Fluor 555 against skeletal muscle (yellow arrow), as well as their cell junctions (red staining; white arrows) (200×). (f) DIF showing positive staining with FITC conjugated C3c antibodies against the acini of the salivary glands (yellow staining; red arrow) and colocalizing with MYZAP in a salivary duct with Alexa Fluor 555 (white arrow; 400×). [Copyright: ©2018 Abreu-Velez et al.]

TABLE 1. DIF autoantibody staining in the oral mucosal structures, compared with the skin and colocalization with DP I-II, ARVCF, and p0071 autoantibodies

DIF						
Antibodies	**Oral Positivity**	**Strength of Staining**	**Positivity to Oral Mucosa Structures**	**Colocalization with ARVCF, DP-I-II, p0071, and MYZAP**	**Positivity in Skin**	**Strength of Staining**
IgG	40/45	(+++)	Epithelial cell junction dot staining. Some stem cells like at the BMZ. The neurovascular bundles and salivary glands including their serous acini and excretory ducts, mainly their cell junctions. Neutrophil extracellular traps. Cell junctions in the dermal connective tissue. Unique individual cells, resembling lymphocytes in shape with "opsonized" features.	100%	40/45	(+++)
Fibrinogen	39/45	(+++)	Intracorneal and subcorneal blisters. Intracytoplasmic and pericytoplasmic staining on keratinocytes (uneven pattern). Dot staining on cell junctions over the entire mucosa. Cell junctions in the dermal connective tissue. Skeletal muscle staining. BMZ of the salivary glands, its serous acini, and the excretory ducts. Neurovascular bundles. Encapsulated neural receptors.	100%	39/45	(+++)
IgM	38/45	(+++)	The mucosal corneal cell layer, dot staining on cell junctions. The BMZ, neurovascular bundles, skeletal muscle, and some of their intracellular organelles. The BMZ of salivary glands, including serous acini and excretory duct cell junctions. Receptors linked with the glands.	100%	38/45	(+++)
Albumin	38/45	(+++)	Mucosal cell junction dot staining. Salivary gland BMZs, their serous acini and excretory duct cell junctions. Cell junctions in the dermal connective tissue. Large neural receptors, colocalizing with JAM-A.	100% and with JAM-A	38/45	(+++)
Complement/ C3c	35/45	(+++)	Cell junctions between keratinocytes. Mucosal BMZ. Neural receptors linked to the salivary glands. BMZ of the salivary glands. Neurovascular bundles. Skeletal muscle cell junctions. Cell junctions in the dermal connective tissue. Unique individual cells resembling lymphocytes in shape with "opsonized" features.	100% and with JAM-A	35/45	(+++)

(Continued next page)

TABLE 1. DIF autoantibody staining in the oral mucosal structures, compared with the skin and colocalization with DP I-II, ARVCF, and p0071 autoantibodies (continued)

DIF						
Antibodies	Oral Positivity	Strength of Staining	Positivity to Oral Mucosa Structures	Colocalization with ARVCF, DP-I-II, p0071, and MYZAP	Positivity in Skin	Strength of Staining
Complement/C1q	35/45	(++)	Cytoplasm of mucosal keratinocytes, patchy; dot staining in the BMZ cell junctions and in the salivary glands and their ducts. Striated muscle and its cell junctions. Neural receptors in the salivary glands. Cell junctions in the dermal connective tissue. Unique individual cells resembling lymphocytes in shape lymphocytes, with "opsonized" features that colocalize with CD3.	100% and JAM-A	35/45	(++)
Complement/C4	17/45		Epithelium, BMZ, and striated muscle.		17/45	
IgA	17/45	(++)	Corneal layer, epithelial dot staining on cell junctions, and pericytoplasmic cell staining in the basaloid layer. Salivary ducts as well as smooth muscle and skeletal muscle, and basal layer cells. Connective tissue cell junctions. Neural receptors in the glands.	100%	39/45	(++)
IgD	16/45	(++)	Skeletal muscle and its cell junctions. Positive on neurovascular supply structures under the BMZ. Positive on ducts of the salivary glands.	100%	16/45	(++)
IgE	7/45	(++)	Receptors in the salivary glands. Unique individual cells resembling lymphocytes in shape, with "opsonized" features that colocalize with CD3.	100%	7/45	(++)
Lambda	40/45	(+++)	Staining on subcorneal blisters and epithelial cell junctions. On salivary glands, including their serous acini and excretory ducts. Skeletal muscle and its cell junctions and on connective tissue cell junctions.	100%	40/45	(+++)
Kappa	40/45	(+++)	Staining on subcorneal blisters and epithelial cell junctions. On salivary glands including their serous acini and excretory ducts. Skeletal muscle and its cell junctions and on connective tissue cell junctions.	100%	40/45	(+++)

IHC Staining

Using metallothionein we observed patchy spot staining at the BMZ, as well as in the neurovascular bundles, the salivary glands including serous acini and the excretory ducts, cell junctions, striated muscle, mesenchymal-endothelial cell junction connective tissue (mainly against the cell junctions), and inside some striated muscle organelles. TIMP1 was positive in the corneal layer and the upper epidermal layers in some patients, and in others in the lower epithelial layers and in the cell junctions of the vessels and cell junctions of the salivary ducts (Figure 3).

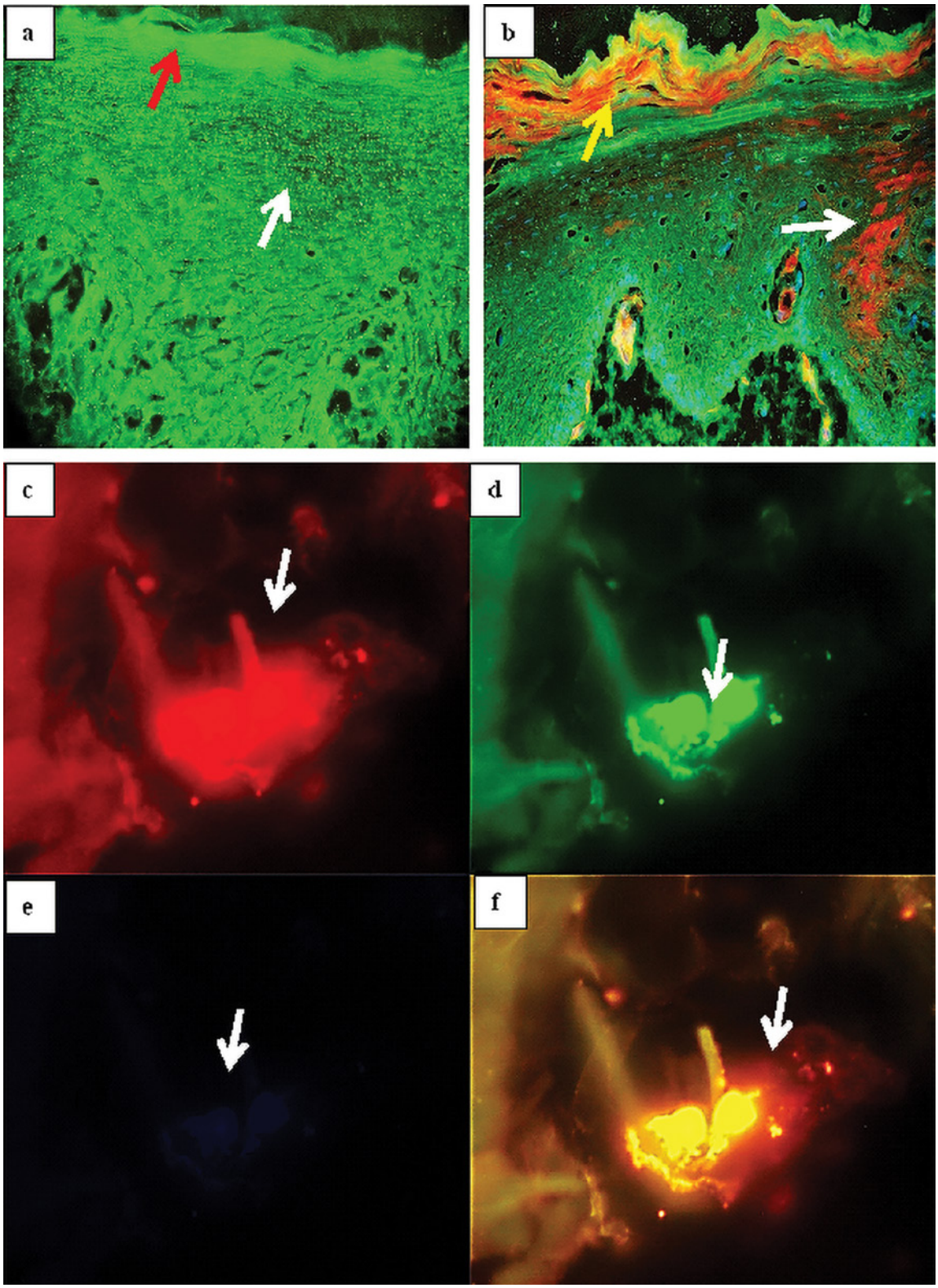

Figure 2. (a) DIF showing positive dot staining with FITC conjugated IgG antibodies against epithelial cell junctions (light green staining; white arrow) (200×) and in the corneal layer (light green staining; red arrow). (b) DIF showing positive staining with FITC conjugated IgG antibodies against the corneal layer (yellow staining; yellow arrow) and dot cell junction staining in epithelial cells (light green staining; white arrow). ARVCF staining with Alexa Fluor 555 is noted in a salivary gland duct (red staining; white arrow) (200×) and in the corneal layer (red staining; yellow arrow). (c-f) Confocal microscopy, using multiple channels of fluorescence. In c, we used antibody to IgM FITC channel (excitation/emission, 495/519 nm); in (d) an antibody to p0071 (Texas red, 555 channel) (excitation/emission, 555/568 nm); in (e) a DAPI channel (blue) (excitation/emission, 360/460 nm); and in f, the combination of all showing a perfect colocalization against neuroreceptors in a salivary gland (in c-f, white arrows, 1,000×). [Copyright: ©2018 Abreu-Velez et al.]

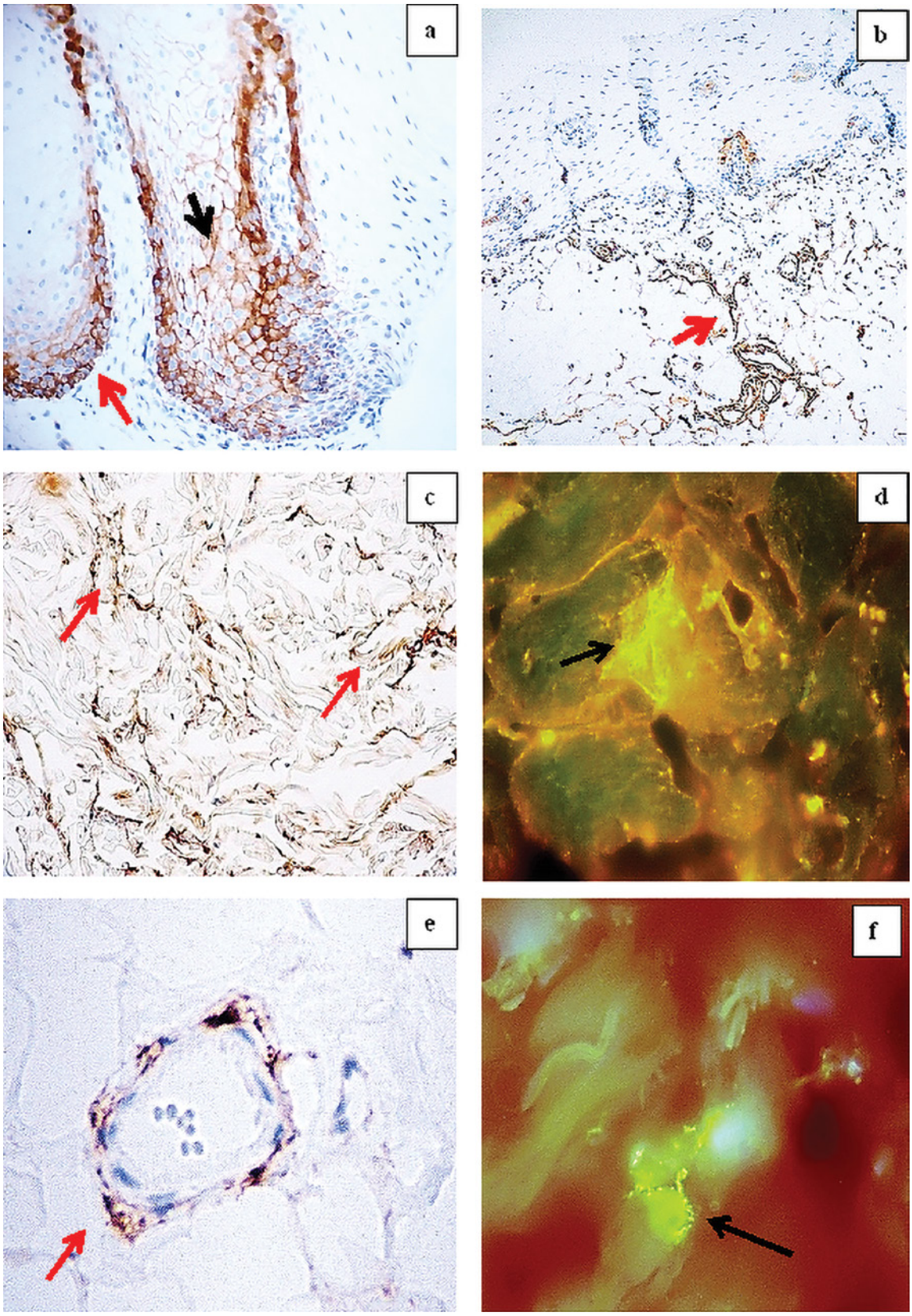

Figure 3. (a) IHC positive staining for metallothionein between oral mucosal cell junctions (black arrow) as well as at the BMZ (brown staining; red arrow; 200×). (b) IHC positive staining for IgA on neurovascular dermal structures (brown staining; red arrow) (100×). (c) IHC positive staining mesenchymal-endothelial junctions in dermal connective tissue cell junctions (brown staining; red arrow)(200×). (d) DIF, showing positive staining with IgA FITC conjugate on dermal connective tissue cell junctions (black arrow) colocalizing with ARVCF conjugate with Alexa Fluor 555 (400×). (e) IHC positive staining with metallothionein in a salivary gland (brown staining; red arrow)(400×). (f) DIF positive staining with FITC conjugated C1q, colocalizing with Texas red DP I-II in the oral mucosa (black arrow, 1,000×). [Copyright: ©2018 Abreu-Velez et al.]

Conclusions

Herein, we report for the first time that patients affected by a new variant of EPF in El Bagre, Colombia, have oral manifestations. The main clinical finding was the asymptomatic loss of teeth, and the main pathological finding was subcorneal blistering. The asymptomatic autoimmune response is directed at multiple structures in the oral mucosa, primarily to cell junctions of the epithelia, dermis, salivary glands, and vessels. We also describe for the first time that the intrinsic oral mucosal immune system, including IgA and secretory IgA, appears to play an important role in this asymptomatic autoimmunity. Previously, we showed that El Bagre-EPF patients have a polyclonal immune response in the skin and against other organs involving IgA [12-15].

In this case-control study we found some clinical alterations such as loss of teeth in the El Bagre-EPF group. Prednisone therapy and lack of oral hygiene can explain these findings. However, the controls have similar demographic factors, with the exception of the intake of prednisone. The autoantibodies to the periodontal ligament could contribute to weakness of the patients' teeth. We previously reported autoantibodies to several smooth muscle structures including the arrector pili muscle in most El Bagre-EPF patients [13].

In theory, clinical involvement of the oral mucosa is not typically present in pemphigus foliaceus. Previously published data indicate that in pemphigus foliaceus, desmoglein 1 (Dsg1) and desmoglein 3 (Dsg3) are expressed in a pathogenic distribution throughout the squamous mucosal epithelia and the skin [16-18]. In our data, we observed something completely different that contradicts this hypothesis, ie, the "theory of Dsg1-Dsg3 compensation."

Measuring salivation in the endemic area is also difficult, but with use of multicolor immunofluorescence [19] we were able to observe positivity to neuroreceptors using high magnifications and color contrast. We also previously demonstrated that the El Bagre-EPF patients have autoantibodies to their palms and soles, as well as to their sweat glands with an IgA response (immune-specific to these anatomic sites) [20,21].

Our findings pointed us to an IgA autoimmune response that is part of the mucosal innate immunity, including the saliva containing lysozymes, bacteriocidins, defensins, cationic proteins, and lactoferrin [22]. Our findings brought our attention to the specific immunity that the oral mucosa has in comparison with the skin. Tomasi and his colleagues in the mid-1960s originally documented oral "local immunity" with the presence of IgA antibodies in secretions including saliva [23-25].

A group of Brazilian authors performed a study of the oral cavity of 56 patients with fogo selvagem (FS). Histopathological and clinical examination of the gingivae of 8 patients revealed FS in the acute and bullous phases of the disease and significant periodontal disease [26].

Other authors studied 15 patients with FS reporting subcorneal acantholysis and no oral blisters or erosions, but DIF demonstrated the presence of tissue-bound autoantibodies in both the epidermis and the oral epithelium of all patients [6].

Other authors studied patients with FS and 4 control subjects, examining the oral mucosa using electron microscopy. In addition to showing clinically normal oral mucosa, electron microscopy showed widening of the intercellular spaces between keratinocytes [6-11].

We observed an autoimmune response to neural receptors in El Bagre-EPF patients' oral mucosa and salivary glands; indeed, we have described autoreactivity to skin neurovascular structures and neural receptors in previous studies [27,28]. The neuronal/transmitter control of salivary glands is performed by both dopaminergic and serotonergic neurons and receptors [29]. Both classes of transmitters elicit saliva secretion. The neurons contain γ-aminobutyric acid (GABA). GABA-positive fibers form a network around most salivary acinar lobules and a dense plexus in the interior of a minor fraction of acinar lobules.

We conclude that patients affected by El Bagre-EPF have an autoimmune response in the oral mucosa. We suggest that this process results in loss of teeth; IgA, and the mucosal immune system seem to play important roles. Given our observations, the Dsg1-Dsg3 compensation theory offered to explain a "lack" of oral compromise in pemphigus foliaceus (including its endemic variants) may need reassessment.

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