

## Endemic pemphigus over a century: Part II

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### Abstract

**Background:** Endemic pemphigus foliaceus (EPF) is an autoimmune disease, classically occurring in a restricted geographic area. Foci of EPF have been described in several Central and South American countries, often affecting young people and Amerindians, with some female predilection. Although most American EPF cases have been documented in Brazil, cases have been reported in Peru, Paraguay, El Salvador and Venezuela. An additional variant of EPF has been described in El Bagre, Colombia, (El Bagre-EPF) affecting older men and a few post-menopausal females. Finally, one additional type of EPF has been described in nomadic tribes affecting females of child bearing age in Tunisia, Africa. **Aims:** The main aim of this review is to summarize current knowledge about autoantigens, and immunologic and genetic studies in EPF. **Material and Methods:** We utilized a retrospective review of the literature, aiming to compile and compare the multiple geographic foci of EPF. **Results:** The primary autoantigens in EPF are still considered to be desmogleins in the case of the Tunisian and all American cases, in contradistinction to plakins and desmogleins in El Bagre-EPF. Although several autoantigens are being suggested, their biochemical nature needs further elucidation. Current knowledge still supports the concept that an antibody mediated immune response represents the principal pathophysiology in all variants of EPF. **Conclusion:** A strong genetic susceptibility appears to contribute to disease development in several people affected by these diseases; however, no specific genes have been confirmed at present. We conclude that further investigation is necessary to define these disorders immunologically and genetically.

**Keywords:** Pemphigus foliaceus, endemic pemphigus foliaceus, fogo selvagem, autoimmunity.

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### Immunological aspects of endemic pemphigus foliaceus

#### General features

Fogo selvagem (FS) has been classically defined as a B cell-mediated disease due to the presence of both 1) spontaneously appearing, intraepidermal clinical blisters as well as 2) epidermal-specific autoantibodies belonging

to the IgG class [1-3], predominantly the IgG4 subclass. FS autoantibody titers correlate with clinical activity and disease severity [1-3]. The role of B cells in endemic pemphigus foliaceus (EPF) is also demonstrated by the demonstration that IgG and the F(ab')<sub>2</sub> Fab' fragments of FS IgG are pathogenic when passively transferred into neonatal mice and rabbits [4-7]. In 1937, a model of FS in rabbits was first developed by injecting the serum of PF patients [6]. These animals developed intra-epidermal blisters, which clinically, histologically, and

immunologically duplicated the human disease [6, 7]. The role of B cells in the disease is also highlighted by the passive transfer of maternal pregnancy IgG autoantibodies (pregnant FS patients with high levels of autoantibodies). The antibody transfer has been reported in less than five cases, with temporary compromise of the fetus. Moreover, the “materno-fetal passive transfer” EPF disease consistently clears over the first two months of life [8]. Thus, this disease is present only temporarily, causing difficulties with any comparison to the natural, chronic course of EPF in adults. No neonatal cases of the El Bagre endemic pemphigus foliaceus (El Bagre-EPF) or the Tunisian variant (Tunisian EPF) have been described, since the disease in these variants does not affect women of childbearing age [9-11].

Although El Bagre-EPF intercellular keratinocyte autoantibodies are primarily from the IgG4 subclass, and their IIF titers correlate with clinical activity and disease severity (as also reported in FS), El Bagre EPF sera contains autoantibodies of the IgG3 subclass [9-11] that recognize an intracytoplasmic antigen, whose nature remains unknown [9,11]. In addition to this potential antigen(s), many others likely remain unknown in El Bagre-EPF disease. Moreover, in addition to plakins, desmogleins and bullous pemphigoid antigens 1 and 3 in El Bagre EPF, studies have shown emerging autoantigens in FS such E-cadherin [12]. The role of classical cadherins as immunological targets of FS was well documented by 1) immunoprecipitation coupled with immunoblotting (IP-IB) and 2) ELISA techniques utilizing a baculovirus-expressed ectodomain of E-cadherin. By IP-IB, anti-E-cadherin reactivity was detected in 15 FS sera. The immunoreactivity of FS sera with E-cadherin was also demonstrated by IP-IB using human epidermal extracts. However, immunofluorescence staining of A431DE cells (E-cadherin positive, Dsg1 negative) with pemphigus sera showed negative results. Immunoabsorption and competitive ELISA analysis suggest that most of the anti-E-cadherin antibodies cross-react with desmoglein 1 (Dsg1), whereas others may represent independent antibodies that do not cross-react with Dsg1. The functional relevance of these anti-E-cadherin IgG autoantibodies detected in these pemphigus sera remains to be defined [12].

The baculovirus heterologous gene expression system has also been utilized to attempt to explain intramolecular epitope spreading on FS [13].

Another autoantigen described in FS is an ubiquitin carrier protein [14]. The authors screened the autoantibodies from a FS patient utilizing a lambda gt11 human keratinocyte cDNA library. One immunoreactive cDNA clone (lambda EPF5) containing a 900-base pair insert was isolated, and subjected to further analysis. Eight of 25 FS sera were then shown to react with an EPF5 fusion protein on immunoblots (IB). The EPF5 cDNA insert hybridized with a 1.2-kilobase epidermal RNA transcript on a Northern blot. Sequence analysis revealed that lambda EPF5 contained a complete coding sequence for a 24-kDa

polypeptide exhibiting significant sequence homology with a family of enzymes known as ubiquitin carrier proteins, or E2s, which are an essential component of the ubiquitin-protein conjugation system [14]. The relevance of the cloned EPF epitope in the pathogenesis of this autoimmune disorder remains to be determined [14].

The same group of researchers also identified a novel IgG anti-keratin autoantibody in the serum of a FS patient (Cascas-42) [15]. The Cascas-42 antibody was shown to be specific for a 59 kDa acidic murine keratin, as well as its 56.5 kDa human counterpart (Moll classification Cytokeratin 10); it was also distinct from previously described pemphigus antibodies. Anti-keratin autoantibodies present in the Cascas-42 serum were purified by affinity chromatography with a 59 kDa murine keratin-agarose column [15].

#### **EPF and autoantibodies**

In 80% of El Bagre-EPF patients, the presence of a “lupus-like band” is seen on direct immunofluorescence (DIF) [9, 10]. The identity of the individual autoantigens, and deposition of a possible immune-complex at the basement membrane zone (BMZ) are not yet characterized. Selected EPF patients have developed 1) glomerulonephritis with chronic renal failure, and 2) sudden death syndrome; however, correlation of these clinical sequelae has not been demonstrated to be associated with EPF. In addition to the lupus band, preliminary studies have revealed the presence of EPF autoantibodies against urinary bladder, parietal cells of the stomach, kidney, neural, vascular, and skeletal, cardiac and smooth muscle cells; however, their role in pathogenicity needs to be clarified (Abreu et. al., manuscript in preparation).

A recent study utilizing FS sera searched for the presence of a broad spectrum of autoantibodies and possible association with other autoimmune diseases in 120 patients with FS and 200 healthy controls from Brazil [16]. In contrast to El-Bagre EPF, no differences were found between the two groups; however, 92.5% of the patients were undergoing steroid therapy. Other studies have also been reviewed [85]; for example, 196 patients with FS from Mato Grosso do Sul and 20 FS cases from Goiás (states of Brazil) were analyzed for the presence of antinuclear antibodies (ANA) by indirect immunofluorescence (IIF), with no positive results [16]. In 28 patients with FS, 21.4% were positive for anti-gastric parietal cell (GPCA) antibodies, 7.1% for anti-mitochondrial antibodies (AMAs), and 3.6% for anti-smooth muscle (SMA) antibodies [16]. However, the autoantibody positivity in these studies was not conclusively diagnostic of other, concurrent autoimmune disease. The 1) use of nonspecific methods, 2) small number of patients investigated and 3) concomitant steroid therapy could contribute to variations in the results obtained [16,17].

#### **Autoantigens and epitope-spreading in EPF**

Desmoglein 1 (Dsg1) is reported to be one of the antigens

recognized by autoantibodies in FS, Tunisian EPF and El Bagre-EPF [9-11, 17-25]. However, we recently reported some differences between FS and El Bagre-EPF by examining autoantigens in both diseases by various biochemical and molecular biological techniques, including immunoblotting (IB), immunoprecipitation (IP) and ELISA; we utilized various antigen sources including baculovirus-expressed proteins [9]. In order to confirm the reactivity of El Bagre EPF sera with plakin family proteins (also known as paraneoplastic pemphigus antigens), we examined sera from both El Bagre-EPF and FS patients by IB using various recombinant, domain-specific peptides [9]. These studies indicated that a considerable number of El Bagre-EPF sera reacted with various domains of periplakin, while only a few FS sera reacted with some of them [9]. Recombinant envoplakin was also recognized by a few sera of both types of EPF. In addition, the number of El Bagre-EPF sera reactive with recombinant periplakin proteins was significantly higher than the FS sera, indicating that preferential reactivity with periplakin may be characteristic of El Bagre-EPF [9]. In contrast, the sera of both types of EPF rarely reacted with BP230 recombinant proteins. Therefore, it seems likely that the 230 kDa protein band detected by some El-Bagre-EPF sera in our previous studies [9-11] may, in fact, represent a different protein with a molecular weight of approximately 230 kDa different than BP230. We also developed an ELISA for detecting a heterogeneous antibody population in serum from people suffering from EL Bagre-EPF that is suitable for detection of plakins [26]. Another interesting finding is that El-Bagre-EPF sera preferentially reacted with paraneoplastic pemphigus (PNP) antigens, and there may be a similar pathogenic mechanism in autoantibody production in El Bagre-EPF and PNP, although the two diseases are quite different clinically and epidemiologically [27,28]. These results lead us to speculate that, in conjunction with genetic predisposition, specific triggers in each endemic focus may induce a highly immunogenic state in the patients, resulting in the production of antibodies to many self-antigens. In addition, we speculate that some of these self-antigens (specifically in El Bagre-EPF cases) are associated with the antigenic response seen in paraneoplastic syndromes [27, 28]. Further, multiple other proteins of different molecular weights are specifically recognized by both FS and El Bagre-EPF patient sera. One possible explanation for these findings might involve an epitope spreading mechanism leading to the production of multiple autoantibodies, although the amino acid sequences of the desmogleins and plakins display marked differences.

Sera from FS patients in the preclinical stage have been shown to recognize epitopes on the COOH-terminal EC5 domain of Dsg1. Actual clinical disease onset is associated with the emergence of specific antibodies for epitopes on the NH2-terminal EC1 and EC2 domains; all sera from FS patients with active disease recognize the EC1 and/or EC2 domains of Dsg1 (21-25). Further, sera from FS patients in remission showed reactivity restricted to EC5. These results suggest that anti-Dsg1 autoantibodies in FS are initially raised against the COOH-terminal EC5 domain in

individuals without skin disease. In genetically predisposed subjects, the autoimmune response may then undergo intramolecular epitope spreading toward epitopes on the NH2-terminal EC1 and EC2 domains of Dsg1, leading to disease onset. Moreover, intramolecular epitope spreading could also modulate remissions and relapses of FS [21-25].

FS is endemic in Limão Verde (LV), state of Mato Grosso, Brazil. In one study, anti-Dsg1 IgM antibodies were detected in sera from 58% of FS LV patients (n=31), in contradistinction to sera from other geographic areas, including 19% of FS patients from Hospital-Campo Grande (n=57), 19% from Hospital-Goiania (n=42), 12% from Hospital-São Paulo (n=56), 10% of PF patients from United States (n=20), and 0% of PF patients from Japan (n=20) [29]. Sera from pemphigus vulgaris patients (PV) (n=40, USA and Japan), bullous pemphigoid patients (BP) (n=40, USA), and healthy donors (n=55, USA) demonstrated negligible percentages of positivity [270]. High percentages of positive anti-Dsg1 IgM antibodies were found in healthy donors from four rural, Brazilian Amerindian populations (42% of 243) as compared with healthy urban donors (14% of 81; P<0.001). More than 50% of healthy donors from LV (n=99, age 5-20 years) possessed IgM anti-Dsg1 across a wide age range, whereas IgG-anti-Dsg1 was differentially detected in age specific ranges: 2.9% at ages 5-10 years, 7.3% at ages 11-15 years, and 29% of donors above age 16. Anti-Dsg1 IgM epitopes were found to be Ca<sup>2+</sup> and carbohydrate-independent [29]. Based on the above data, the authors suggested the possibility that anti-Dsg1 IgM antibodies are common in FS patients in their native environment, and uncommon in other pemphigus phenotypes as well as in FS patients who migrate to urban hospitals. Thus, recurrent environmental antigenic exposure may lead to both IgG and IgM responses that trigger FS [29].

Another FS study utilized FS hybridomas that secreted either IgG or IgM (predominantly IgG1 subclass) autoantibodies from the B cells of 1) eight current FS patients, as well as 2) one individual taken 4 years before FS onset; the H and L chain V genes of anti-Dsg1 autoantibodies were analyzed [30]. Multiple lines of evidence suggest that these anti-Dsg1 autoantibodies are antigen selected. First, clonally related sets of anti-Dsg1 hybridomas characterize the response in individual FS patients. Second, H and L chain V gene use seems to be biased, particularly among IgG hybridomas, and third, most hybridomas are mutants and exhibit a bias in favor of CDR (complementary determining region) amino acid replacement(AAR) mutations [30]. Pre-FS hybridomas also exhibit evidence of antigen selection, including an overlap in V (H) gene use and shared multiple AAR mutations with anti-Dsg1 FS hybridomas, suggesting selection by the same or similar antigen. The authors concluded that the anti-Dsg1 response in FS is antigen driven, and that selection for mutant anti-Dsg1 B cells begins well before the onset of disease [30].

The prevalence of anti-Dsg1 antibodies in healthy subjects

and their distribution in the different regions of Tunisia was recently investigated, to better identify endemic areas of pemphigus foliaceus (PF) [25]. The authors tested, by ELISA, sera from 270 normal subjects recruited from different Tunisian areas, 90 Tunisian EPF patients and 203 healthy relatives these patients. Seventy-six patients (84.4%), 20 healthy controls (7.4%), and 32 relatives (15.76%) possessed anti-Dsg1 antibodies [25]. In southern Tunisian regions, where EPF is associated with a significant sex ratio imbalance (9 females: 1 male in the south vs. 2.3: 1 in the north) and a lower mean age of disease onset (33.5 years in the south vs. 45 years in the north), a higher prevalence of anti-Dsg1 antibodies in healthy controls was observed (9.23% vs. 5.71% in the north). The highest prevalence of anti-Dsg1 antibodies in healthy relatives (up to 22%) was observed in the most rural southern localities. More than half anti-Dsg1 positive healthy controls were living in rural conditions with a farming occupation, which suggests that this activity may expose the subjects to predilecting environmental conditions [25].

In addition, desmocollins I and II have been reported as autoantigens recognized by sera from some patients with FS [31]. Desmocollins are transmembranous glycoproteins that serve as components of desmosomal junctions, and occur in three different isoforms (desmocollins 1, 2 and 3); all isoforms are represented in the epidermis [31]. In this study, the authors examined sera of various types of pemphigus by immunoblotting (IB) with purified bovine desmosomes and bovine desmocollin 1, 2 and 3 fusion proteins, expressed in pGEX vectors. Six of 15 (40.0%) FS sera, two of 18 (11.1%) non-endemic pemphigus foliaceus sera, eight of 39 (20.5%) pemphigus vulgaris (PV) sera, and two of 11 (18.2%) normal sera, demonstrated reactivity with desmocollins from desmosomes. Experiments with the fusion proteins showed that no desmocollin isoform was specifically recognized by sera of any individual type of pemphigus. The authors concluded that the pathogenesis of pemphigus might thus be more complex than previously believed [31].

#### **Mechanisms involved in acantholysis in EPF**

Few studies have reported information regarding the mechanisms of acantholysis in EPF. Exposure of uninvolved skin to UVB induces acantholysis, with *in vivo* binding of IgG and C3 to the epidermal intercellular spaces (32). Nevertheless, it is also known that complement activation *per se* is not necessary for pemphigus bulla formation [32]. Recently, the role of proteases in the pathology of acantholysis in pemphigus has been addressed [33, 34]. Since 1983, the plasminogen-plasmin system has been implicated in the pathophysiology of EPF [34]. However, mitigating against the participation of this system in acantholysis, pemphigus vulgaris and pemphigus foliaceus autoantibodies were shown to be pathogenic in plasminogen activator knockout mice [35]. Nevertheless, other work has suggested that regulation of the expression of the urokinase receptor and junctional proteins (including plasminogen, its activators,

and the kininogen-kallikrein-kinin system) contribute to acantholysis as part of the mechanisms mediating pemphigus [36].

The role of urokinase type plasminogen activator (uPA) has been well documented in the pathogenesis of pemphigus vulgaris (PV) [37, 38]. Activation of plasminogen into the active serine protease plasmin initiates extracellular proteolysis, leading to acantholysis; however, the mechanisms underlying this process are not clearly understood. In addition, the expression of p63 and the induction of apoptosis have been documented in healthy and lesional skin from FS patients, when compared to normal subjects [39]. In a separate study, an IgG passive transfer mouse model of PF was utilized to investigate the relevance of the apoptotic mechanism in pemphigus pathogenesis. TUNEL-positive (apoptosis positive) epidermal cells and increased oligonucleosomes in epidermal cytosolic fractions were detected in diseased mice. A time course analysis revealed that the TUNEL-positive epidermal cells appeared prior to the formation of intra-epidermal blisters. Moreover, after PF IgG injection, the pro-apoptotic factor Bax was upregulated at early time points (2 and 4 h), and the anti-apoptotic factor Bcl-x<sub>L</sub> was downregulated at later time points (6, 8, and 20 h) (determined by Western blot analysis). The active forms of caspase-3 and -6 (a putative initiator of acantholysis) were detected in the later time period (6, 8, and 20 h). In addition, administration of Ac-DEVD-cmk, a peptide-based caspase-3/7 inhibitor, protected mice from developing intra-epidermal blisters and clinical disease induced by PF IgG. The same protective effect was also observed utilizing a broad-spectrum caspase inhibitor, Bok-D-fmk. Collectively, these findings show that biochemical events leading to apoptosis are provoked in the epidermis of mice injected with PF autoantibodies. Caspase activation may directly contribute to acantholytic blister formation in PF [40].

Other studies in FS have shown that mannose-binding lectin (MBL) and MBL-associated serine protease (MASP-2) play a key role in innate immunity; their deficiency has also been related to both increased susceptibility to infection and development of autoimmune diseases not related to FS [40]. MBL and MASP-2 serum levels were measured in 114 patients with FS and in 100 healthy individuals in Brazil [41]. MBL and MASP-2 levels were measured by sandwich assays (time-resolved immunofluorimetric assays) utilizing monoclonal antibodies. No differences were observed in MBL levels in patients with FS compared with controls [mean SEM 1230.07, +/- 132.18 ng/mL (median 789.0 ng/mL) vs. mean 1036.98, +/- 117.99 ng/mL (median 559.5 ng/mL), *p* = 0.32]. Non-significant, lower MASP-2 levels were observed in EPF patients, compared with controls [mean 274.34, +/- 15.66 ng/mL (median 239.5 ng/mL), vs. mean 304.72, +/- 15.28 ng/mL [median 261.0 ng/mL), *p* = 0.06]. MBL deficiency (< 10 ng/mL) and MASP-2 deficiency (< 100 ng/mL) did not differ significantly between patients and controls. These data indicate that MBL and MASP-2

deficiency are not associated with susceptibility to FS [41].

### **The role of B and T lymphocytes, dendritic cells, chemokines and cytokines in EPF**

Few studies have been performed testing immune cells other than B cells in EPF. In recent studies, B and T lymphocytes were quantified in the peripheral blood of 30 FS patients according to their ability to form rosettes with 1) sheep erythrocytes, or 2) sheep erythrocytes sensitized with antibody and complement [42, 43]. The total T lymphocyte count and the functional T cell count were significantly lower (by Student's t test) as detected by the active rosette test [42, 43] in FS versus normal patients. Significantly, the FS patients were not receiving any immunosuppressive medicine when tested [42, 43]. In addition, peripheral lymph nodes from three patients showed a decrease in the number of T cells in the paracortical areas [42, 43]. Paradoxically, it had been thought that B cells represented the main immunological mediators in the pathogenesis EPF. However, multiple complications of EPF are associated with non-B-cell immunity, including secondary infections by 1) poxvirus (*Molluscum contagiosum*), 2) *Mycobacterium tuberculosis*, 3) parasites (*strongyloidiasis* and *entamoebiasis*), 4) fungi and 5) herpes virus, including the "relapsing Kaposi's varicelliform eruption". Thus, these studies suggest that FS is not solely a B-cell-mediated disease [42, 43]. It is important to keep in mind that susceptibility to these secondary infections may or may not be related to concurrent corticoid therapy. Further, an understanding of the immune response of patients to molluscum virus may also help to explain the susceptibility to smallpox seen in some individuals.

An additional study was conducted on 16 patients with FS, ten of them with the localized form (group G1) and six with the disseminated form (group G2). These patients underwent complete blood counts, quantitation of mononuclear cell subpopulations by monoclonal antibodies, studies of blastic lymphocyte transformation, and quantitation of circulating FS antibodies by IIF testing. The test profile was created order to correlate their clinical signs, symptoms and laboratory data with their immunological profiles and to determine the relationship between circulating autoantibody titers, lesion intensity and course of lesions under treatment. Leukocytosis was observed consistently, especially in group G2. All patients showed decreased relative CD3+ and CD4+ T cell values, and a tendency towards decreased relative values of the CD8+ T cell subpopulation. Blastic lymphocyte transformation indices in the presence of phytohemagglutinin were higher in patients of groups G1 and G2 than in controls. The FS IIF testing was positive in 100% of G2 patients and in 80% of G1 patients. The median value for the IIF antibody titers was higher in group G2 than in group G1. Analyzing the results, the authors concluded that cell immunity was preserved, and that there was a direct relationship between antibody titers detected by the IIF test and dissemination of skin lesions [44]. Other authors have reported that the immune

response is preserved in FS [45].

To elucidate the role of pathogenic T cells in FS autoimmunity, T cell receptors (TCRs) were characterized utilizing complementary DNA isolated from 17 Dsg1-specific T cell clones, generated from FS patients by clonal expansion in vitro [46]. To analyze the T cell repertoire, a panel of primers, collectively specific for the known human T cell receptor  $\beta$  variable region (TCRBV) family, was paired with a constant region primer in order to amplify one distinct T cell receptor  $\beta$  variable region allele for each T cell clone [46]. Polymerase chain reaction (PCR) products were then sequenced to determine the precise  $\beta$  chain gene usages. In the 17 clones tested, 10 distinct T cell receptor  $\beta$  variable region usages and nine TCR  $\beta$  joining gene segment usages were identified. Furthermore, TCR  $\beta$  variable region and  $\beta$  joining usage did not appear to be random, but were oligoclonal in nature, with some preference shown for T cell receptor  $\beta$  variable regions 5S1 and TCR BJ2S5 [46].

In addition, another study characterizing the autoimmune T cell response associated with FS indicated that the great majority of FS patients have circulating T lymphocytes that specifically proliferate in response to the extracellular domain of Dsg1. Long-term T cell cultures developed from these patients also responded to Dsg1, and this antigen-specific response was shown to be restricted to HLA-DR molecules. These Dsg1-reactive FS T cells exhibited a CD4-positive memory T-cell phenotype, and produced a T helper 2(Th2)-like cytokine profile [47]. Other studies have shown that if tissue damage is mediated by anti-Dsg1 antibodies in EPF, an initial T cell response is a likely requirement for autoantibody generation [47].

Further studies have described attempts to determine the number of dendritic Langerhans cells in FS [48]. Since serum IL-12 is increased in patients with FS and Langerhans cells (LC) produce IL-12, serum IL-12 autoantibodies were titrated by Elisa in another study, resulting in increased level in PF sera [52]. Biopsies of blistering lesions were obtained from 22 FS patients, 13 of whom were submitted to biopsy of both lesional and apparently healthy skin. The control groups consisted of skin from 8 cadavers, and from 12 unaffected women presenting for breast plastic surgery. LCs and DCs were also identified with anti-CD1a antibodies, and quantified by morphometric analysis. LC numbers in the lesional and non-lesional skin from FS patients were similar to that of both control groups. DC numbers in the lesional skin (median=0.94 DC/mm basement membrane) were higher than those of the cadaver group (median=0.13 DC/mm basement membrane). In the 13 FS patients with biopsies of both lesional and non-lesional skin, LCs and DCs were present in larger numbers in the lesions. A direct correlation existed between DC numbers in the lesions of the FS group and serum autoantibody titers [48]; this correlation was not observed for LC numbers. The increased number of DCs in the lesion, as well as the direct correlation with serum autoantibody titers suggests the participation of DCs in the pathogenesis of PF. The

relationship between increased DC number and IL-12 in PF needs to be further clarified [48].

Some attention has been given to the role of dendritic cells in the mechanism of EPF acantholysis [49]. A predominance of CD4+ T cells, a decrease in Langerhans cells (LCs), an increase of CD1a+ dermal dendritic cells, and a lack of keratinocyte ICAM-1/CD54 and HLA-DR molecules in lesional skin was observed in FS [49]. Twenty biopsy specimens of lesional and perilesional skin of FS patients were analyzed by immunohistochemical techniques. The panel of monoclonal antibodies included CD1a, CD4, CD8, HLA-DR, IL-2R, LFA-1/CD11a, ICAM-1/CD54, and PAN-B. A semiquantitative analysis of the cell populations revealed a predominance of CD4+ T lymphocytes in the tissue in perilesional and lesional skin. The population of LCs was decreased in lesional skin when compared with the perilesional skin, whereas CD1a+ dermal dendritic cells predominated in lesional skin. Keratinocyte expression of ICAM-1/CD54 and HLA-DR was negative in both lesional and perilesional skin. These results suggest the involvement of cell-mediated immunity in FS. The lack of keratinocyte ICAM-1/CD54 expression may be related to the pattern of cytokines secreted by the CD4+ T cells of the tissue in FS [49]. Utilizing morphometric analysis, increased dermal dendritic cells in lesional skin from EPF patients was directly correlated with IgG autoantibody titration by IIF. These results may reflect the possibility that impairment in the cellular, and/or more complex immune response might play an important role in FS disease [49]. Other authors had reported paucicellularity of S-100 positive cells in perilesional and lesional skin from patients with FS [50]. In 36 FS patients, the presence of S-100 protein antigens identified by immunohistochemistry showed a marked reduction or even disappearance of the cell population bearing the S-100 antigen in lesional skin [50]. These results are in agreement with the findings discussed above, and might provide an indirect insight into the possible "peripheral anergy" that may be part of the loss of peripheral tolerance in EPF disease [50].

A recent study evaluated the expression of the proinflammatory cytokines interleukin 1, interferon gamma and tumor necrosis factor alpha; the pro-apoptotic inducers Fas and inducible nitric oxide synthase; and the apoptosis inhibitor Bcl-2 to evaluate the presence of apoptosis in FS [51]. Skin biopsies from 13 patients with FS and controls were evaluated by immunohistochemistry, and apoptosis was determined by a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay. The results showed that proinflammatory cytokines were only detected in cells of the inflammatory exudate. Inducible nitric oxide synthase, Fas, and Bcl-2 were all expressed by both epithelial and inflammatory cells [51]. Epithelial apoptosis was observed in 12 cases (92.3%), and subepithelial apoptosis in 11 cases (85%) [51]. The study suggests that apoptosis, as well as a local production of proinflammatory cytokines are associated with FS lesions. These results may contribute to the development of new therapeutic approaches to FS [51].

Another study in FS investigated levels of serum cytokines. Twenty-five patients with FS and a control group consisting of 10 healthy individuals were studied [51, 52]. Serum IL-2, IL-4, IL-5, IL-10, IL-12 and IFN-gamma were measured in the two groups by ELISA. The median concentration of IL-2 was lower in PF patients compared to the control group (0.45 and 9.50 pg/ml, respectively), as was the concentration of IL-4 (0.26 and 10.16 pg/ml, respectively). The same was observed for IL-5 (7.94 and 15.74 pg/ml, respectively) and for IFN-gamma (5.90 and 8.58 pg/ml, respectively). For IL-10 and IL-12, higher concentrations were observed in FS compared to the control group (IL-10: 24.76 and 20.92; IL-12: 2.92 and 1.17 pg/ml, respectively). The authors concluded that considering the Th1/Th2 paradigm, it seems that a Th2 profile, largely manifested by IL-10, predominates in FS [52]. Another study reported that supernatants of peripheral blood mononuclear cell populations from patients affected by FS produced significantly more interleukin-1beta (IL-1beta) than those from stimulated healthy controls. Furthermore, a Th2 bias was observed in FS patients when the IL-5/gamma interferon ratio was analyzed. These results indicate that cells from FS patients react with a vigorous proinflammatory response [51, 52].

One group of researchers studied the association of routine laboratory tests in FS, and screened 20 patients for antinuclear antibodies (ANAs), rheumatoid factor (RF), C-reactive protein (CRP), and changes in the erythrocyte sedimentation rate (ESR), serum proteins electrophoresis (SPEP) and total leukocyte count. The CRP was found to be elevated in 60% of FS cases, a leukocytosis in 85%, and an elevated ESR and mild alterations in the serum protein electrophoretic analysis in all of the patients. No ANA or RF changes were found. Although widely accepted as nonspecific tests, the authors believe that an association of these routine laboratory tests with the clinical findings can prove to be helpful in the follow-up care of FS patients [53].

#### **Cutaneous hypersensitivity mediated by IgE in EPF**

In addition to the immunological alterations mentioned above, skin hypersensitivity mediated by IgE has been thought to play a role in the pathogenesis of FS. The concept is based on studies utilizing a reverse reagenic test in patients with FS (16 patients), and an equal number of epidemiologically matched controls living in the same endemic area [54]. In both groups, skin tests were performed using house dust, air fungi, and *Dermatophagoid sp.* dust mite antigens. All patients and controls were selected to be negative for a history of atopy. The IgE levels in patients with FS were significantly increased when compared with the control group [54]. Another study also showed that patients with El Bagre-EPF exhibited increased IgE levels compared with controls [54]. We recently reported an elevation of IgE levels in serum of patients with El Bagre-EPF utilizing a case controlled study, and this increase correlated with high levels of mercury in the hair and nails of El Bagre-EPF patients [55].

### Complement and EPF

The complement system represents one of the major mediators of inflammation and the humoral immune response, which when activated evokes multiple biological effects. Multiple studies have shown that complement may be an important mediator of skin lesions in pemphigus [56-66]. Although complement does not seem to be essential for acantholysis, its activation appears to amplify the pathogenicity of pemphigus autoantibodies. Specifically, in FS, it has been shown that autoantibodies activate complement components “*in vitro*”, and that complement activation “*in vivo*” is strongly associated with disease activity. One of our authors has demonstrated by DIF that FS patients display simultaneous intercellular deposition of C3 and IgG autoantibodies; and that 57% of the patients in one study presented C3 deposition in the skin basement membrane zone [59, 65]. Circulating pemphigus autoantibodies displayed titers ranging from 1:10 to more than 1:1280, and the titers were drastically decreased during treatment [59, 65]. Although complement may not be absolutely necessary for the development of skin lesions in EPF, these longitudinal studies showed that significant activation of complement is observed during the active phase of the disease [59, 65].

### Lesional and perilesional skin and mucosae in EPF

Research utilizing DIF in FS has been primarily focused on the study of lesional and perilesional skin, while little attention has been given to uninvolved skin. In a recent study, the authors analyzed the frequency of IgA, IgM, and IgG (and its subclasses IgG1, IgG2, IgG3 and IgG4) and C3 complement fraction deposition in the intercellular spaces (ICS) and basal membrane zones (BMZ) in uninvolved, lesional and perilesional skin from 47 FS patients with DIF [66]. The results showed a predominance of IgG and IgG4 deposition in all skin samples, followed by C3 and IgG1 deposits. The positive response for IgG in uninvolved (91.48%), lesional (93.61%) and perilesional (97.87%) skin was similar to that found for IgG4 in the same samples: 95.74%, 95.74% and 97.87%, respectively. Regarding IgG1, the uninvolved skin showed lower results (14.89%) than the lesional (29.78%) and perilesional skin (29.78%). With C3, the perilesional skin displayed higher results (40.42%) than the uninvolved and lesional skin (34.04% for both) [66]. The results suggest the importance of uninvolved skin for direct immunofluorescence in the diagnosis of FS, and further suggest that any cutaneous region can demonstrate pemphigus antibodies by DIF [66].

Other authors studied a group of 16 patients with chronic FS under corticosteroid therapy and persistent erythematous, papulous, verrucous, and generally hyperpigmented lesions, which were characterized as corticoid therapy resistant lesions [67]. The study was conducted utilizing anatomic pathology and DIF. Pathologically, the lesions showed tendencies toward epithelial hyperplasia, and cleavage in variable levels of the epidermis. These findings differ from classic, early

lesions of FS, and contain features of chronic FS lesions examined in the pre-corticoid therapy era. The DIF of the injured skin was positive for IgG in 93.75% of these persistent cases, similar to the early stages of FS; the IgG was negative in a single persistent case in which there was no epidermal cleavage. In addition, in eight of the persistent lesion patients, the DIF and IIF of the healthy skin were studied. The DIF was positive in three of these FS cases, and the IIF was negative in all eight [67].

Other authors investigated whether ultrastructural changes present in clinically normal mucosa could occur in patients with FS. Surgical biopsy specimens were taken from the foreskin of 8 patients with EPF and 3 control subjects, from the uterine cervix and vaginal wall of 9 patients with FS and 2 controls, and from the oral mucosa of 5 patients with FS and 4 controls [68]. The patients all had received a clinical and histopathologic diagnosis of EPF, and all had clinically normal oral and genital mucosa. In the FS patients, widening of the intercellular spaces and distended, elongated cytoplasmic projections (the tips of which contained desmosomes that were sometimes disassembled), were evident in all 4 regions studied [68]. At the edges of the spinous layer keratinocytes, cytoplasmic vesicles were present, apparently containing intact or fragmented desmosomes, or half-desmosomes: These ultrastructural findings in the mucosa are similar to those previously described in the literature in the oral mucosa of patients with FS. In the current FS study, although the desmosomal changes occurred in all epithelial layers, blisters did not occur in the mucosa, possible due to a coexpression of desmoglein 1 and desmoglein 3 [68].

Often in FS, no clinical lesions are seen in the mucosa and non glabrous skin; however, some additional studies have shown disease autoreactivity in these areas [69-73]. In El Bagre-EPF, some of the reactivity is directed towards the sweat glands, the palms, neurovascular areas, the meibomian glands and tarsal muscle. Clinical lesions in the eyes were historically reported by Arneondola [69].

### Mouse models in EPF

Early studies of EPF utilizing animal models that were reported in 1937 by Lindenberg [6], and by Beutner et al. in 1971 [7]. Further, multiple studies using passive or active, largely neonatal mouse models have been described in the study of EPF [3-5]. It has been noted that the presence of EPF autoantibodies is not sufficient to clinically present or maintain the disease, since the passive transfer experiments on animals using EPF autoantibodies often resulted in temporary disease if the animals were clinically followed [3-5].

One animal FS passive transfer model has been utilized to examine the possible pathogenic role of FS autoantibodies. IgG from the sera of human FS patients was purified, and injected into the peritoneum (IP) of neonatal BALB/c mice [3-5]. Thirty-four of 46 mice (74%) receiving parenteral IgG fractions from FS patients developed cutaneous lesions that were identical to the human disease by clinical,

histologic, immunologic, and ultrastructural criteria. High-titer FS sera produced lesions more consistently and more rapidly than low-titer sera. When the injections were discontinued, new lesions ceased to appear and old lesions resolved. The extent of murine disease directly correlated with the titer of human anti-epithelial antibodies detected in the mouse serum. Similar concentrations of IgG fractions obtained from sera of human controls from endemic and non-endemic areas did not induce disease when injected into littermates of the diseased mice. The same researchers subsequently demonstrated that monovalent Fab' immunoglobulin fragments from FS autoantibodies can reproduce the human disease in neonatal BALB/c mice [3-5].

Another study has shown that FS acantholysis occurs independently of IL-12, since one of us have shown experimental pemphigus in mutant C57BL/6 animals null for IL-12, in spite of increased levels of serum IL-12 found in FS patients [74].

Some FS mouse models have been studied utilizing electron microscopy (EM) [75]. The dynamic ultrastructural changes of FS IgG-induced acantholysis in mice were studied. FS IgG was injected IP into neonatal BALB/c mice. Skin and serum were studied at 0, 1, 3, 6, 12, 18, and 24 hours post injection by immunofluorescence (IF), electron microscopy, and immuno-EM. Binding of FS IgG in the intercellular spaces (ICS) of the basal cell layer was seen by IF within 1 hour and was strongest at 12 hours. IgG binding affected the spinous and granular cell layer by 12 hours, then faded and remain localized only in the basal cell layer at 24 hours. By immuno-EM, IgG binding was diffuse along the keratinocyte surface. Edema of the ICS in the basal cell layer was present at 1 hour by EM. At 12 h, there was formation of microvilli, with intact desmosomes at the tip of the microvillous projections. Splitting of desmosomes (forming half desmosomes) and acantholysis (primarily affecting the granular cell layer) were most prominent between 12 and 24 hours. The plaques of the half desmosomes then gradually disappeared, and their tonofilaments retracted into the keratinocyte cytoplasm. Detaching keratinocytes demonstrated cytoplasmic vacuolizations, swollen mitochondria, and internalizations of both intact and half desmosomes (remnants of split desmosomes) [75]. The investigation showed that the ultrastructural changes observed in the epidermis of patients with FS can be duplicated in experimental animals by IP injection of FS IgG. Further, FS IgG may have direct effects on the assembly and disassembly of desmosomes [75].

## Genetic aspects of FS

FS occurs among rural Brazilians living in geographically clustered disease foci, perhaps suggesting that people who carry the proper genetic background develop the disease under the influence of some unknown environmental stimuli. One of the most important features regarding EPF is the fact that at least in the FS and El-Bagre-EPF variants,

one third of the patients have relatives affected by the same disorder [1, 9, 10]. In addition, other, unaffected relatives exhibit autoantibodies in their sera that recognize some of the EPF antigens (e.g., Dsg1) [1, 9, 10], but these autoantibodies do not seem to play a sufficient role in the immunopathogenesis of either FS or El-Bagre EPF. Currently, a non-classical Mendelian association has been found relative to EPF, and more complex genetic segregation studies and studies of allelic interactions must be performed to understand the mechanisms underlying the pathogenesis of this autoimmune disease. One important point is that in both the Brazilian areas where FS has been prevailed and the El-Bagre area, interracial mating between Amerindians and Europeans has occurred over a relatively short span of time. The ancestry of the Amerindian groups also seems to differ within South America. Multiple tools have been utilized to study the migratory patterns of both the Amerindians, as well as historical immigrants into the "New World" [76]. In FS, familial cases are frequent, and not everyone living in the endemic region develops this disease, suggesting that host factors play a role in determining whether exposed individuals will be affected (76). Previous studies with FS patients indicate the possibility that some HLA alleles confer an increased risk for the development of EPF disease [77-79]. Some studies have also shown a possible association between FS itself and HLA alleles. Brazilian caucasoid FS patients and matched controls were tested for HLA-A, B, C; DR1 to DRw8, and DQw1 to DQw3. The frequencies of DR1, DR4, and B16 were significantly increased, while DR7 was significantly decreased among the FS patients (156-158). Given these findings, it was suggested that at least two MHC-class II genes are involved in the pathogenesis of FS. Two alleles, namely DQw1 (associated with DR1), and DQw3 (associated with DR4), confer susceptibility to FS. In contradistinction, at least one allele, DQw2 (associated with DR7, DQw2 and DR3) confers resistance [77-79]. The susceptibility gene(s) (if allelic) seem(s) to be epistatic to or dominant over the resistance gene(s). Other studies had shown the alleles DRB1 (\*) 0101, (\*) 0102, (\*) 0103, (\*) 0404, (\*) 0406, (\*) 0410, (\*) 1406 and (\*) 1601 to be significantly more frequent among patients with FS [77-79].

One genetic study has shown a positive association between FS and selected cytokine genetic variants. The study samples included 168 patients and 189 controls, and were comprised of mostly Caucasians and Mulattos. The approach consisted of a case-control association study, and the alleles were identified by mismatched PCR-RFLP. No associations were found with the cytokine genetic variants IL1A, IL1B, IL1RN, IL4R and IL10. There was a weak negative association of the haplotype -1082G -592C (OR=0.49) with the IL10 gene in Mulattos. In regards to polymorphism -590 of the IL4 gene, a positive association with the T/T genotype (OR=2.71) and a negative association with the C variant (OR=0.37) were found. Associations with IL6 -174 variants suggest that the C/C genotype has a protective effect (OR=0.13), while carriers of the G allele are more susceptible (OR=7.66) to FS [80]. Other studies from the same author have shown no

association between the polymorphisms of the CTLA4, tumor necrosis factor and lymphotoxin- $\alpha$  genes and FS [81, 82].

A recent study reported the presence of EPF in two native Yanomami children from the Venezuelan Amazonia; the children presented with erythroderma, were hospitalized, and subsequent clinical, histologic, and immunofluorescence studies diagnosed EPF [82]. An analysis of human leukocyte antigen class II showed the DRB1\*04 subtype \*0411, which has not been previously associated with this disease. However, it shares a common epitope with all the HLA DRB1 alleles that have been involved in this disease among Brazilian populations [83.]. Contradictory results have also been reported when studying the pemphigus foliaceus and desmoglein 1 gene polymorphism [84, 85].

Other authors determined the HLA DR/DQ markers of susceptibility and protection in Tunisian EPF [24]. Genomic DNA from 90 patients with PF recruited from all parts of the country and matched by age, sex and geographical origin with 270 healthy individuals was genotyped. First, when the whole patient population was studied, DRB1\*03, DQB1\*0302 and DRB1\*04 alleles were significantly associated with the disease, while a significant decrease of DRB1\*11 and DQB1\*0301 were observed in patients compared with controls. DRB1\*0301 was the dominant allele in DR3-positive patients and controls, while DRB1\*0402 was found in 42% of DR4-positive patients. Second, when the HLA DR/DQ allele distribution was studied after dividing patients according to their geographical origin, the southern group (which consisted exclusively of patients with the endemic form of the disease) showed the same associations as the whole PF population, particularly with DRB1\*03. In the northern group, only the DRB1\*04 and DQB1\*0301 alleles were found to be associated. Interestingly, anti-desmoglein 1 antibody-positive healthy controls did not carry susceptibility alleles; in contrast, most carried negatively associated alleles. These observations indicate that a particular genetic background characterizes Tunisian EPF; and further, that HLA class II genes control the pathogenic properties of the autoimmune response, rather than the initial breakdown of B-cell tolerance [24].

A recent study of El Bagre-EPF featured Complex Segregation Analysis (CSA) and analysis of short tandem repeats (STRs) to discriminate between environmental and/or genetic factors in this disorder [86]. The CSA analysis was carried out according to the unified model; implemented utilizing the transmission probabilities implemented in the computer program POINTER, and evaluated using a software package for population genetic data analysis (GDA), namely Arlequin. The authors also performed pedigree analyses utilizing Cyrillic 2.1 software, with a total of 30 families and 50 probands (47 males and 3 females) tested. In parallel to the CSA, the authors also tested for the presence of short tandem repeats from HLA class II, DQ alpha 1, involving the gene locus D6S291 by using the Hardy-Weinberg- Castle law [86]. The results

indicate that the best model of inheritance in this disease is a mixed model, with multifactorial effects presenting within a recessive genotype. Two types of possible segregation patterns were found: one with strong recessive penetrance, in families whose phenotype is more Amerindian-like, and a second of possible somatic mutations. The authors noted that the penetrance of 10% or less in female patients 60 years of age or older indicates that hormones could protect younger females. The greatest risk factor for men being affected by El Bagre-EPF was the NN genotype. These findings are only possible due to somatic mutations, and/or strong environmental effects. The authors also found a protective role for two genetic loci (D6S1019 and D6S439) in the control group [86].

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