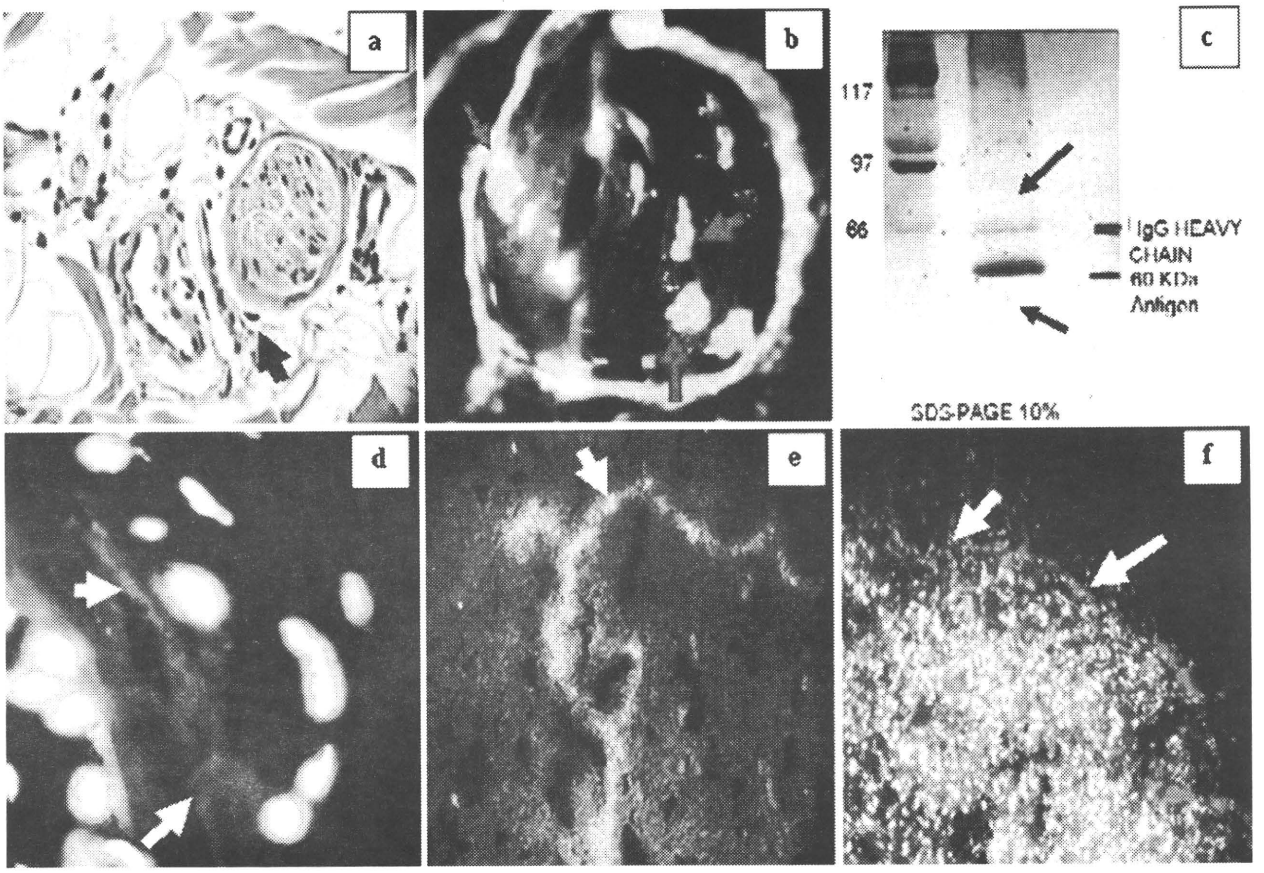


Fig. 4 El Bagre-EPF patient sera recognize myelinated and non-myelinated nerves, the spindle cell apparatus, and some brain tissues by different techniques

Bagre-EPF sera reactivity to several bands are shown, including 135, 97, 66 and 60 kDa(the last two, highlighted with blue arrows, were correlated using anti-human-IgG and IgM, respectively). We found that one third of the El Bagre-EPF sera were reactive to some of these four bands. No controls from outside or inside the endemic area were positive. Figure 4e illustrates positive FITC-conjugated IgG from an El Bagre-EPF patient, showing positive reactivity to the rat brain gyral and the sulci surface (green staining; white arrow). In Fig. 4f, similar pattern is seen with DPI-II (red staining; white arrows). We were able to demonstrate that El-Bagre-EPF patient autoantibodies colocalized in several areas of the brain and neurovascular structures with the commercial antibodies from Progen directed to DPI and DPII, to ARVCF, and with the P0071 antibodies (data no shown).

In Fig. 5, El Bagre-EPF patient autoantibodies colocalize with neural markers using IEM, CFM, and IHC. Figure 5a, c demonstrates colocalization of the neural components with El Bagre-EPF patient sera using FITC-conjugated human IgM and IgA antibodies (white staining; yellow arrows) and Texas red-conjugated GFAP (orange staining) (red arrows). In Fig. 5b, El Bagre-EPF autoantibodies colocalize using FITCI-conjugated human-IgM antibodies (yellow staining; red arrows). In Fig. 5d, e, CFM demonstrated that the El Bagre-EPF antibodies colocalized with neural markers. Figure 5d shows CFM image revealing anti-GFAP (red), anti-IgG (green), and Dapi (nuclei; blue) in their respective staining patterns, utilizing the EZ 1 Viewer software for image analysis. The light gray represents the overlapping of GFAP and IgG using NDIC. In Fig. 5e, we measured the staining of the overlap distances and found colocalization of the dyes in the same focal plane. The GFAP and the IgG peaks overlap (white arrows), in contrast to the Dapi (yellow arrow). Figure 5f, g shows IEM images. We observed 10 nm gold particles, representative of El Bagre-EPF autoantibodies deposited in the axons of the peripheral nerves at lower magnification (Fig. 5f, 60 kV) and at higher magnification (Fig. 5g, 150 kV) (red arrows, black particles).

Table I shows mercury levels in the patients and controls and documents neural alterations found in comparison with mercury levels at the time of the examination.

Discussion

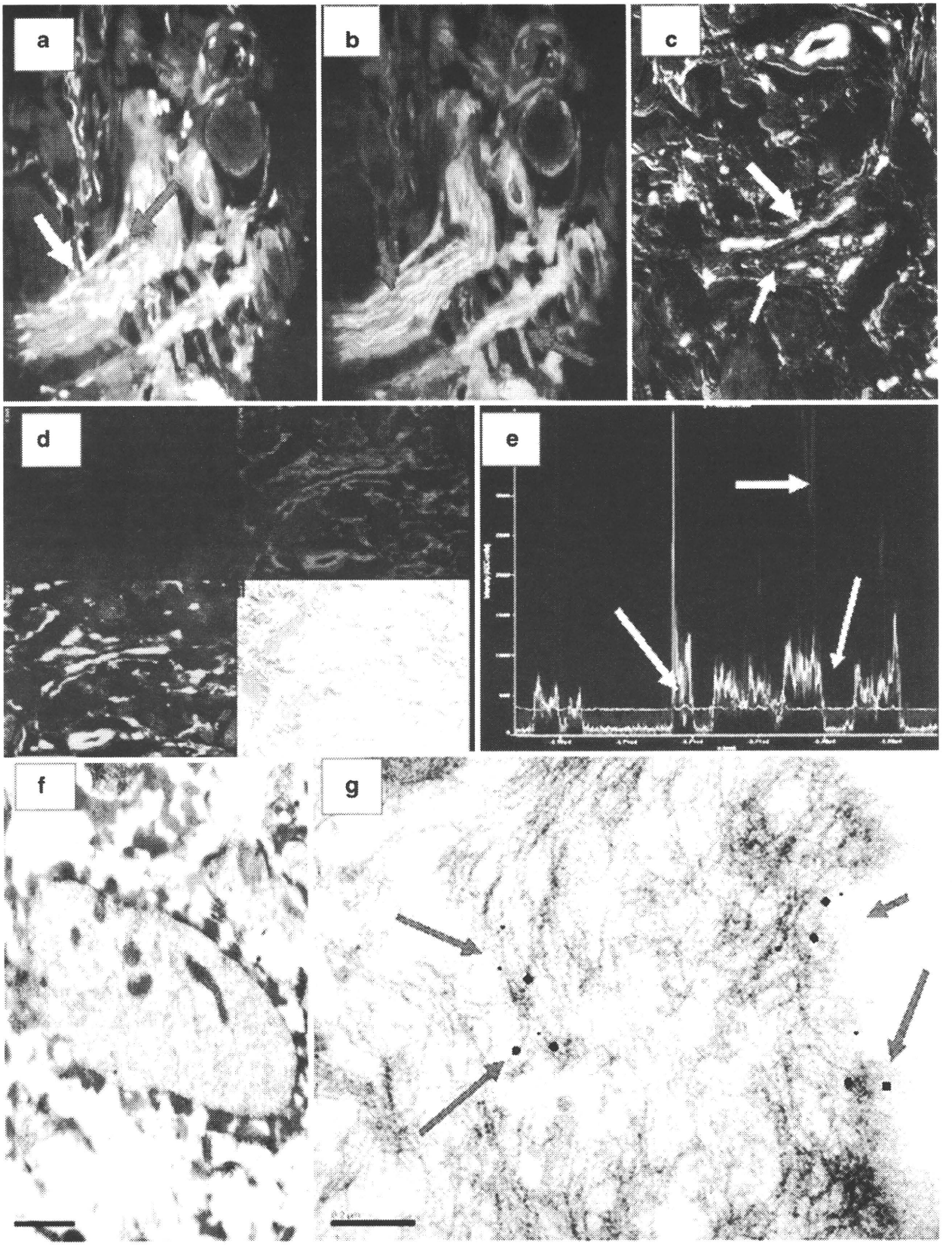
Patients affected by FS and El Bagre-EPF have burning sensation on the skin, combined with itching and occasional paresthesias statistically significant even comparing with the controls living in the same endemic area where mercury and other metals and metalloids prevail [4, 5, 7, 18]. Other neuromuscular symptoms reported before the steroid era for patients affected chronically by EPF included depression, mood disturbances, decalcifications, muscular atrophy (most of extensors muscle), contractual deformities, and ankylosis of the joints producing the "pose of pemphigus" (dosiflexion of extremities) [1–8]. Based on our studies, we can speculate that the "pose of pemphigus" is explained vis-a-vis for the weakness and/or direct damage of the extensor nerves for an unknown reason to us yet [1–12]. Our findings of polyclonal neural reactivity by colocalization of El Bagre-EPF autoantibodies with neurovascular markers could explain the skin burning and "be in fire sensation" symptoms and could provide valuable clues to the pathophysiological process. In addition, antibodies to the spindle cells of the neuromuscular apparatus may explain the clinical neuromuscular atrophy and the increased dorsiflexural tone in the "pose of pemphigus". Furthermore, by IB, we detected reactivity of several molecules, including plakins and unknown molecules of 135, 97, 66, and 60 kDa in several sera from El Bagre-EPF patients. Of interest, p0071 is 135 kDa in molecular weight, and ARVCF approximates 97 kDa. IB revealed that IgM and IgG antibodies correlated with our DIF, IIF, and IHC testing.

We detected (1) autoantibodies to mechanoreceptors and mostly to thin nerves of the skin including some myelinated and some not. We also detected autoantibodies to the optic nerve, but mostly to the perineural meningeal sheaths [that are rich in desmoplakins (El Bagre-EPF antigens)]. In addition, we detected neural paucicellularity, decreases in the ENFD, and autoreactivity to parts of the brain that all colocalized with the patient's autoantibodies and several neural markers. We demonstrated colocalization of the patient's autoantibodies with DPI and DPII (known to be antigens for El Bagre-EPF) in the optic nerve and brain tissues. We recently described several ocular abnormalities and the presence of autoantibodies to the meibomian and other structures of the eyes in El Bagre-EPF patients [6]. Desmosomes are major intercellular junctions found in association with intermediate filaments in epithelial, cardiac, and arachnoidal tissue that surrounds the optic nerve [19]. DPI and DPII are part of the desmosomal plaque and seem to have a role for linking intermediate filaments in several tissues [19]. DPI and DPII are not restricted to stratified epithelia [19]. Interestingly, ARVCF is associated with E-, M-, and possibly N-cadherins [20]. Thus, we suggest that epitope spreading could occur in the disease process in El Bagre-EPF patients.

Within the skin, we observed defragmentation and alterations in the neural plexuses by several methods. Skin biopsy has been demonstrated to be of value in the diagnosis of clinical small fiber neuropathies; this technique is less invasive than nerve biopsy [12–15]. In autonomic neuropathies, diagnostic fibers are located in the dermis; thus, fibers innervating sweat glands and piloerector muscles can be assessed [12–15]. We recently described autoantibodies to sweat glands in patients affected by El Bagre-EPF [21]. Here, we demonstrate antibodies to multiple neurovascular areas. Thus, we have demonstrated physical and immunological evidence of autoreactivity to the peripheral and central nervous system using multiple techniques. However, the neural symptoms could result from a combination of sympathetic and parasympathetic nerves, both myelinated and non-myelinated damage, and therefore, larger and extended studies need to be pursue to answer these questions.

We speculate that damage to the nerves may occur because intraepidermal nerves exist in proximity to desmosomes; blisters, acantholysis, and separation would occur in these areas, potentially exposing neural antigens to autoreactivity. A similar process could occur at the neurovascular plexus below the basement membrane zone or within the dermal papillae. Additionally, mercury, other elements, and/or other diseases could induce epitope mimicry and/or alter the conformation of selected molecules, thus triggering the autoimmunity. p0071 is very homologous to the neural plakophilin-related armadillo repeat protein (NPRAP/δ catenin) and is located in several cell junctions [22]. p0071, DPI, and DPII are part of the complexus adherens meshwork, related to endothelial and lymphatic cells [23]. These cells are connected by the complexus adherens, with VE-cadherin joining with DPI and DPII, as well as several adherens junction plaque proteins, such α- and β-catenin, p120 catenin, and components of tight junctions, such claudin-5, JAM-A, and ZO-1 [23].

Most current literature and trends have clearly showed that most autoimmune skin blistering disease, such pemphigus and pemphigoids, are for the known desmogleins and BP18 and BP230. However, alterations of the nerves or other neural structures have been previously described in bullous diseases and loss over time, including a patient with paraneoplastic pemphigus (PNP) with a pseudotumor of the spinal nerve [24, 25]. Another case of PNP was associated with a myofibroblastic tumor [26]. Two Russian studies have shown alterations in adrenergic and cholinergic nerves of the skin in patients suffering chronic pemphigus [27, 28]. Changes in the Gasserian ganglion have been identified in cases of oral pemphigus [29]. Kaposi reported that many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◀ **Fig. 5** Immunoelectron microscopy reveals deposits of El Bagre-EPF patient autoantibodies within nerve axons, and CFM and IHC demonstrate colocalization with neural markers

cases of fatal pemphigus showed anatomic changes of chronic myelitis in the spinal cord and/or in the sympathetic nerves [30]. Li et. al. reported that sera from a patient with BP with neurological changes recognized BP antigens by IB in the skin and brain [31]. In mice lacking the BPAG1 gene, neurons exhibited perturbations in their intermediate filaments and microtubules, leading to swellings and changes in the axons [31]. Our studies demonstrated autoantibodies by IEM using 10-nm gold particles in the axons of peripheral nerves; further IEM studies will be performed on the central neural system. Additional evidence of the importance of the neural system in pemphigus has been demonstrated by the detection of human alpha-acetylcholine and cholinergic receptors as pemphigus vulgaris antigens [32, 33].

Chronic exposure to mercury produces neural tissue alterations, as seen in acrodynia Minamata disease and in animal models exposed to mercury [34–36]. Symptoms typically include sensory impairment (vision, hearing, and speech), ataxia, disturbed sensation, and a lack of coordination. The type and degree of symptoms exhibited depend upon the individual toxin, the dose, and the method and duration of exposure [34–36]. These alterations differ from the severe burning sensation seen in El Bagre-EPF patients. Organic mercury affects primarily sensory peripheral nerves, with swelling and degeneration of Schwann cells and changes in both myelin sheaths and axons [32–35] Also differing, the skin biopsies from El Bagre-EPF showed several inflammatory cells as reported before and showed here [5, 8]. Most of autoantibodies produced by chronic mercury exposure alone in human and animals have been reported to be antinucleolar, differing to our findings.

In El Bagre-EPF patients, we have documented autoantibodies in the axons by IEM. Since autoantibodies to neural structures following exposure to mercury, metalloids or organophosphates are primarily directed against MBP and GFAP and of IgG, IgA, and IgM subclasses, a mercury association cannot be excluded in El Bagre-EPF etiology [34–36]. However, mercury exposure alone is not sufficient to develop pemphigus. In Minamata disease, no patients have been described with EPF [34–36]. In addition, in the control group from the endemic area, the predominance of any autoreactivity was seen using IgM and present only in some individuals; in contrast, most of the neurological alterations and the skin burning sensations presented exclusively in El Bagre-EPF patients. Finally, the autoantibodies in the patients were expressed against perineurium,

Table I Mercury levels in the patients and controls and neural alterations found in comparison with mercury levels at the time of the examination

Subjects	Disease Course	Range of levels of mercury detected in nails and hair in parts per million (ppm) where (+ mild, ++ moderate and +++ severe)	Presence of autoantibodies to neural structures, and isotypes of the antibodies	Skin burning sensation	Kinesiological and neurological alterations
El Bagre-EPF cases	Acute (<6 months after disease onset)	40% (+++) 20% (++) 20% (+) 20% (5+) 0% (0)	None	Positive in 100% cases	Fine tremor 50%
El Bagre-EPF cases	Chronic cases(>3 years after disease onset)	40% (+++) 20% (++) 20% (+) 20% (5+) 0% (0)	IgM alone (40%) IgA alone (40%) IgA, IgM, fibrinogen, C3 and IgG (70%)	Positive in 100% cases	Depression (100%)
Controls living in the endemic area	<6 months	30% (+++) 20% (++) 10% (+) 20% (5+) 20% (0)	None	Negative in 100% cases	Fine tremor 50%
Controls living in the endemic area	>3 years	40% (+++) 20% (++) 20% (+) 20% (5+) 0% (0)	IgM 1 control	Negative in 100% cases	Depression (30%)
Controls from out of the endemic area		100% (0)	None	Negative in 100% cases	None

epineurum, endoneurium, and blood vessels Both the NHP and the LKS clinical evaluation scale results correlate with our findings [16, 17]. We note that most of our affected nerves innervated extensor muscles; the significance of this finding is beyond the extent of our current study.

A paraformaldehyde prefixation of skin biopsies improved our assessment of neural tissues; we suggest larger studies to assess technique efficacy. Furthermore, the El Bagre-EPF patient focus exists in a rural area, with minimal medical resources. Ideal testing would address specific antigens with neural microarrays, quantitative sensory nerve testing (for large and small fibers), quantitative sudomotor axonal reflex testing, single photon and positron emission tomography, and electromyography.

Plakins are the major El Bagre-EPF antigens [1–12]. Homozygous mice with a desmoplakin gene ablation and subsequent rescue utilizing extra-embyonic ectoderm have shown that desmoplakin is important in embryonic development, affecting heart, neuroepithelium, skin, and vasculature integrity [37, 38]. Finally Balo and Foldavari [38] reported in 1948 that in the Gasserian ganglia and in other spinal ganglia, a chronic inflammatory process with perivascular infiltration, vacuolar degeneration of ganglionic cells, and proliferation of amphicytes [38] occurs. In addition, there is the presence of some granulomas, which were found at the juncture of posterior roots and spinal ganglia. These and other authors speculated that the disturbance of fluid circulation is caused by the granulomas consisting of arachnoidal cells. Our findings of autoreactivity against the arachnoid envelope antibodies may explain these findings. In summary, we suggest that neural autoreactivity may contribute to burning skin sensations, paresthesias, and the "pose of pemphigus" encountered in patients with EPF.

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