Detection of Mercury and Other Undetermined Materials in Skin Biopsies of Endemic Pemphigus Foliaceus

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Abstract: A novel variant of endemic pemphigus foliaceus (EPF) was described among individuals in an area surrounding El Bagre, Colombia, South America. The population in this rural mining community is exposed to high environmental levels of mercury, used for gold extraction, as well as other minerals, metalloids, and trace elements (e.g., quartz, rutile, granite, magnetite, and almenite) and ultra-violet radiation. Fifty control subjects and fifty EPF patients in the endemic area were examined for the presence of mercury in skin biopsies and hair, using autometallographic and mass spectroscopic analyses, respectively. Simultaneously, serum levels of IgE were measured, and cutaneous tests for hypersensitivity reactions were performed. Using autometallography, mercuric sulfides/selenides were detected in 14 of 51 skin biopsies distributed similarly in the control and patient groups. However, significantly higher serum IgE levels and mercury concentrations in hair, urine, and nails were found in patients compared with controls. Microscopic analysis revealed mercuric sulfides/selenides concentrated within and around the sweat gland epithelium, as well as in dendritic cells. Five skin biopsies from EPF patients and five from controls that tested positive for the presence of mercuric sulfides/selenides by autometallography were randomly selected for electron microscopic analysis. This analysis revealed a mixed electron-dense and electron-light material closely associated with desmosomes in patients. However, there were intracellular vesicles containing an amalgam of electron-dense and electron-light materials only in the EPF patients. Thus, EPF-affected individuals are exposed to high levels of environmental mercuric sulfides/selenides and other elements. This is the first study reporting mercuric sulfides/selenides in skin biopsies from people living in a focus of EPF, and these compounds may play a role in the pathogenesis of autoimmunity.

Key Words: Endemic pemphigus foliaceus, Cell-adhesion molecules, Trace elements, Musculoskeletal diseases, Desmosomes, Autoimmunity, Bio-elements, Mercury

A difficulty in the study of autoimmunity is the identification of the possible triggering factor(s) involved in the process.1 In terms of this issue, endemic pemphigus foliaceus (EPF) is the only described endemic disease observed in a relatively well-defined geographic region.2-3 This unique feature makes EPF an excellent model system to study the complex interactions between genetics, environment, and the immune system.2 EPF has been described in foci in the South American rain forest, mainly in Brazil,2-4 but also in other countries of Latin America (e.g., Colombia)5-9 and in Tunisia.10,11 Although in this latter focus no epidemiological studies were performed, it is generally accepted that pemphigus foliaceus (PF) and Brazilian EPF, or fogo selvagem (FS), are characterized by acantholysis and blistering in the upper epidermal layers with deposits of predominantly IgG4 autoantibodies in the intercellular spaces.12 One major target antigen in these diseases is desmoglein 1 (Dsg1), a desmosomal glycoprotein that belongs to the cadherin family of calcium-dependent cell adhesion molecules, ubiquitously localized in desmosomes.13,14 The cause of FS in Brazil is unknown; however, environmental risk factors, such as exposure to Simulium pruinosum, have been implicated as possible triggering factors for this disease in a host with the proper genetic background.3,14 Another focus of EPF has also been described in the area around El Bagre, Colombia, South America.2,5,7 Additional studies from our laboratory resulting from a 10-year epidemiological survey in El Bagre, Colombia, established that this focus in fact represents a new type of EPF with clinical manifestations similar to those
described for pemphigus erythematosus, better known as Sec- 
early-Usher syndrome (a disease resembling a mixture of lupus 
and pemphigus). El Bagre EPF predominantly affects 40- to 
60-year-old males, as well as a few post-menopausal females, 
and the patients are primarily miners who also engage in agri-
cultural activities. In contrast, Brazilian EPF, or fogo selva-
gem (FS), primarily affects children and young adults, with 
the highest incidence at 10 to 30 years of age, and with both sexes 
equally affected. The El Bagre EPF has the following clinical 
features: (1) hyperpigmented plaques and macules mainly 
on the face and trunk, predominating in the mid-chest area; (2) 
an erythematous butterfly-like macular lesion with fine scaling 
and erosions on the face; (3) pruriginous or hyperkeratotic 
patches with crusting mainly on the presternal and interscapular 
seborrheic regions and armpits; (4) occasional scarring and 
scabbing of the scalp, resembling a false tinea amiantacea; (5) 
hyperpigmentation of previous lesions (seen mostly in people 
demonstrating clinical control); (6) the presence of pustules 
around scaling lesions; and (7) an actinic conjunctivitis-like 
condition. This last feature appears mainly in cases with high 
clinical and immunologic activity. Histopathologic changes in 
lesions of El Bagre EPF mostly resemble those seen in pem-
phigus foliaceus (Abreu et al, manuscript in preparation). Sub-
jects who fulfilled most or all of the above clinical criteria and 
have immunopathologic findings consistent with pemphigus 
were diagnosed with El Bagre EPF (Abreu et al, manuscript in 
press).

Metalloids, minerals, and trace elements (e.g., quartz, 
rutile, granite, magnetite, and almenite) are ubiquitous in El 
Bagre, and in particular, mercury is used daily for gold extrac-
tion processes. Mercury has been demonstrated to trigger au-
toimmune phenomena in rats and mice that possess the proper 
genetic background. Therefore, we searched for the pres-
ence of mercuric sulfides/selenides in the skin and hair of pa-
tients from the rural mining community of El Bagre to deter-
mine a possible correlation between mercury exposure and/or 
its presence in skin and the development of EPF. Mercury has 
also been shown to increase serum IgE levels in genetically 
predisposed rats and mice, which can then develop autoimmu-
nity. Therefore, serum IgE levels were examined to identify 
possible connections to the immune hyper-reactivity seen in 
EPF.

METHODS

Study Subjects

All patients participated freely in this study and signed a 
consent form. In addition, the Institutional Review Board and 
Human Assurance Committee approved this study in accord-
ance with the Scientific and Ethics committees of the respec-
tive institutions. A careful history was obtained from all pa-
tients and controls. During examinations of subjects, clinical 
diagnosis of any skin diseases or allergic processes, as well as 
a complete medical history, were obtained by a trained derma-
tologist and by a trained allergist. Two skin biopsies were 
taken from each subject; in the case of visible dermatitis and/or 
pemphigus lesions, the biopsies were taken in part from le-
SIONAL skin and in part from normal-appearing skin. Most bi-
opsies were taken from the chest area. In the controls with no 
skin lesions, the biopsies were also taken from the chest area, 
and then fixed in 10% formalin. These samples were processed 
by routine H & E staining. The clinical diagnosis was con-
firmed by histopathological analysis by a trained dermatopa-
ologist.

Since it has been demonstrated that some dermatoses can 
increase percutaneous absorption of mercury, we included 
controls with other skin diseases (25 of 50) as well as individu-
als with no dermatoses. Therefore, in addition to 25 normal 
individuals, the following disease controls were used: atopic 
contact dermatitis (5 of 50), actinic keratosis (7 of 50), lichen planus 
(1 of 50), vitiligo (5 of 50), cutaneous amyloidosis (1 of 50), 
basal cell nevus syndrome (1 of 50), nodulocystic acne (1 of 
50), and psoriasis (4 of 50). Patients and controls were matched 
by age, gender, living area, and work activities. Skin biopsies 
were sent to the Department of Oral Pathology, Malmo 
University, Malmo, Sweden for autometallographic analysis 
and to the Department of Dermatology at Wayne State University 
for electron microscopic analysis. Serum was also collected 
and stored at −80°C until analysis.

Mercury Detection in Hair, Urine, and Nails

Since mercury can be toxicologically detected in hair, 
nails, and urine with similar confidence, we measured mercury 
in the hair of all participants and in the nails and urine of half of 
them. After extensive washing, 25 mg of hair and/or nails were 
cut and degreased with acetone. Hair and nail samples were 
packed separately in ash-free paper and incubated in a solution 
of nitric and sulfuric acid followed by a solution of potassium 
permanganate to destroy organic material. Excess potassium 
permanganate was removed with hydroxylamine chloride. 
Following this, tin chloride was added to extract the mercury 
from the hair or nails. Mercury was measured in an atomic 
sorption spectrophotometer (MAS 50). The World Health 
Organization (WHO) accepts a permissible level of mercury in 
hair of 7 parts per million (ppm.)

The total mercury content in urine was determined by 
mass spectrometry with the so-called cold vapor method after 
on-line oxidative treatment of the sample in a microwave 
oven. Use of a KBr/KBrO3 mixture, microwave digestion, 
and a final oxidation with KMnO4 assures complete recovery 
of the organic forms of mercury. Quantitative recoveries were 
obtained for phenyl mercuric chloride, dimethyl mercury, mer-
curic acetate, and methyl mercuric chloride. The determined 
normal limit was similar to that described for hair.
Quantification of Total IgE Serum Levels

Since mercury can increase serum IgE levels in genetically predisposed rats and mice, which can then develop autoimmunity, we tested for serum levels of this molecule in the study subjects. Using a commercial ELISA kit (AlaSTAT Total IgE kit, Diagnostic Products Corporation, Los Angeles, CA), normal IgE levels in rural areas of underdeveloped countries is defined as 300 international units per liter (IU/L) or less. Since parasitic diseases are one of the most common causes of IgE elevations in people living in rural areas, all subjects received anti-parasitic medicines 2 and 4 weeks before testing for serum IgE levels. Serum tests were performed after stool stool tests negative for parasites.

Cutaneous Test

Atopy is another common cause of an increase in serum IgE levels. We tested for a cutaneous response to different allergens by injecting Dermatophagoides (D. pteronyssinus and D. farinae). Approximately 20 to 30 ul of these agents (Abell-loALK, Madrid) at a concentration of one biologic unit were applied intradermally (1 UI/ml I.D.) in the lateral area of the arm, using an insulin syringe. Positive (histamine chlorohydrate 1:10,000) and negative (saline solution) controls were also used. A positive result was indicated by erythema with a diameter of greater than 5 mm and/or edema.

Autometallography

To detect mercury in skin, the method of Danscher and Møller-Madsen was used to visualize tissue-bound mercury in paraffin sections. Briefly 3-µm sections were coated with 0.5% gelatin, air dried, and subsequently developed at 26°C for 1 hour in the dark. The developer contained gum arabic, sodium citrate buffer, hydroquinone, and silver lactate. The gelatin-coated sections were washed in 40°C tap water and rinsed in distilled water, followed by incubation in 5% sodium thiosulfate to suppress background staining. Sections were prepared in quadruplicate, one of which was lightly counterstained with hematoxylin and eosin. The autometallographic technique reveals silver sulfides/selenides, metallic silver, and gold in addition to mercuric sulfides/selenides. To differentiate between mercury and silver, additional sections, mounted on poly-L-lysine-treated glass slides (Polysine, Menzel, Germany), were incubated in a 1% aqueous potassium cyanide (KCN) solution for 2 hours to remove silver prior to autometallographic development.

Electron Microscopic Analysis

Specimens were fixed in 5% glutaraldehyde and 1% osmic acid in phosphate buffer, pH 7.2, and embedded in araldite. The thin sections were stained with 1% uranyl acetate and lead citrate and examined in a Hitachi H-300 electron microscope as previously described.

Statistical Evaluation

Previously codified original data, tabulated in the EpInfo program for distribution frequencies and associations, were graphed with the Harvard Graphics package.

RESULTS

Serum IgE and Mercury Levels in Hair, Nails, or Urine

In all cases, EPF patients showed significantly higher levels of both IgE and mercury levels than the control group (P < 0.05) (Fig. 1). Patients for whom mercury levels were also determined in nails or urine (a 24-hour collection), demonstrated a high correlation with mercury levels measured in hair (Fig. 1). Although a slight increase in mercury levels of the group of controls with dermatoses was observed compared with controls with no dermatosis, this difference was not statistically significant.

Autometallographic Analysis

Our results showed that in 7 biopsies from EPF patients and in 7 controls mercuric sulfides/selenides were visualized as a dark staining. In the 7 positive skin biopsies from controls, 1 belonged to a patient with psoriasis, 1 to a patient with oral lichen planus, and 1 to a patient who had suffered from severe mercury intoxication. The most common localization of mercuric sulfides/selenides was in the secretory epithelium of the sweat glands (Fig. 2 A, B, and C). The skin biopsy from the control patient who had previously suffered from mercury intoxication showed numerous mercuric sulfide/selenide-loaded dendritic–like cells throughout the dermis. A similar positive epidermal staining was observed at the basement membrane in both patients and controls. Most studies using metallographic analysis have been done in Caucasians because it can be difficult to differentiate the melanin pigment from the mercuric selenides/sulfides after staining with silver-based stains. Nevertheless, mercuric selenides/sulfides were observed using the autometallographic technique, in which incubation with 1% aqueous potassium cyanide (KCN) solution for 2 hours to remove silver prior to autometallographic development is performed. Thus, these 2 tests allowed for the simultaneous detection of melanin and mercuric selenides/sulfides.

Mercuric sulfides/selenides were also detected in separate serial sections mixed with melanin grains inside melanophage-like cells in both the epidermis and the dermis. A great number of such cells were detected around the blood vessels and around the sweat gland structures. In addition, mercury was detected inside of sebaceous gland cells.

Cutaneous Test

Only 1 EPF patient tested positive for D. pteronyssinus and D. farinae. In addition, no statistically significant differ-
ences were observed in terms of personal or familial history of allergy or atopy (respiratory, skin, or gastrointestinal reactions) between EPF patients and controls.

Electron Microscopic Analysis

Five EPF and five control skin biopsies that showed mercury in the skin by autometallography were examined by electron microscopy. We observed a mixture of electron-dense and electron-light materials amalgamated in the intercellular spaces of the keratinocytes, mainly at the granular layer, in the 5 EPF patients. These deposits were in contact with the intercellular junctions, where one of the autoantigens in pemphigus foliaceus (desmoglein-1) is located (Fig. 3 A through F). We observed 2 predominant types of vesicles: one inside of the cells and another abutting to the intercellular space. These vesicles contained 2 or 3 unconventional electron-dense and electron-light materials and were clearly different from other physiologically known dense bodies. Serial sections of different magnifications are shown in Figure 2 (Fig. 2 A through F). In the skin biopsies from controls that also tested positive for mercury by autometallography, no alterations in the epidermis were detected by electron microscopic analysis.

DISCUSSION

Endemic clustering of autoimmune diseases following xenobiotic exposures reinforces the hypothesis that these diseases might be secondary to genetic, hormonal, and environmental factors. While the existing correlation between autoimmunity and environmental agents has not yet been well established, some possible predisposing environmental agents have been implicated, including mercury, iodine, vinyl chloride, canavanine, organic solvents, silica, L-tryptophan, particulates, trace elements, ultraviolet radiation, and ozone. Mercury is commonly used to amalgamate gold, thereby facilitating its extraction during mining activities. Mercury also amalgamates multiple trace elements and can compete with calcium in calcium-dependent proteins in an allosteric manner.

Desmogleins are also calcium dependent and may be affected by mercury. This metal, as well as other minerals, metalloids, and trace elements, is also present in many products, such as fertilizers, pesticides, and herbicides, employed in farming activities in this endemic area of pemphigus. The El Bagre area also has special environmental conditions, such as its acidic soil, large amounts of waste ash products from forest
fires, wide jungle deforestation, extremely high temperatures and humidity, thunderstorms, and microbe-mineral-metalloid interactions.15 These factors may increase environmental levels of mercury and other heavy metals, metalloids, minerals, and trace elements.23 Indeed, previously we reported that 64.2% of the patients affected by EPF were exposed to mercury, and perhaps other trace elements, directly by mining or indirectly by fumigation products that contain mercury.5,6 In addition, mercury pollution has been reported in the biotic chain, including humans, in El Bagre and neighboring municipalities.15 The Brazilian FS focus shares several environmental features with the El Bagre EPF focus, such as severe deforestation and extensive mining of rich geological resources.15,29,30 Our study revealed increased levels of mercury in hair as well as the presence of mercuric selenides/sulfides in sweat glands. Thus, we hypothesize that mercuric selenides/sulfides, minerals, metalloids, and other trace elements could be involved directly or indirectly in triggering EPF disease in a genetically predisposed individual. Alternatively, mercury exposure may increase the incidence of a late mutation of some molecules after exposure to chronic ultraviolet radiation, as occurs in some strains of mice.32 Patients with EPF have disruption of the skin barrier, and increased mercury uptake has been observed in the skin of patients with various types of dermatitis, such as active atopy, psoriasis, and lichen planus.18

Regarding the localization of mercuric sulfides/selenides in skin, it was previously reported that in normal and eczematous skin, 10 to 15% of this metal could be absorbed after topical application. After skin exposure, mercury can be absorbed since the incubation with KCN removes such staining. Positive epidermal and basement membrane staining for melanin was observed in both patients and control individuals. However, dihydroxyphenylalanine (DOPA staining) was negative in these cells in the dermis (data not shown). No cluster of differentiation markers were examined in the dendritic cells in the dermis by immunohistochemistry. Thus, the identification of these cells is not possible. D, Specimens in which mercuric selenides/sulfides (arrows) were visualized using autometallography were counter-stained with H & E and photographed at 200× magnification. E, Using autometallographic analysis counter-stained with H & E, a group of melanocyte-like cells around the sweat glands are shown filled with melanin and mercuric selenides/sulfides (arrows) (400×). F, This picture is similar to panel E, but photographed at higher magnification (400×). Fine deposits of mercury are observed inside the sweat gland epithelium (arrows). Panel (G) shows two types of deposits disclosed by autometallographic staining: a finer grained material spread throughout the sebaceous gland (small arrow) and a larger grained and more localized material (large arrow) within individual cells (400×). H, The entire superficial dermis close to the basement membrane area contained abundant dendritic-like cells filled with melanin and/or mercuric/selenides (arrows) (100×). Panel (I) shows similar dendritic-like cells between keratinocytes (arrows) (630×).
detected extracellularly, usually closely associated with desmosomes and external surfaces of cell boundaries. We detected a mixed material with an electron density similar to that of mercury in close proximity to the desmosomes, where one of the target antigens of pemphigus disease is located. As many proteins, like metallothionein and lipoproteins, are detected extracellularly, usually closely associated with desmosomes and external surfaces of cell boundaries. We detected a mixed material with an electron density similar to that of mercury in close proximity to the desmosomes, where one of the target antigens of pemphigus disease is located. As many proteins, like metallothionein and lipoproteins, are detected extracellularly, usually closely associated with desmosomes and external surfaces of cell boundaries. 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promote IgE secretion.\textsuperscript{39} Another study also showed increased serum levels of IgE in patients with FS, but an additional skin test using air fungi was performed and resulted in a slightly elevated cutaneous response in the FS group compared with the controls.\textsuperscript{37} In our study, we did not test for the presence of fungi or fungi-produced toxins, which can also increase IgE serum levels, as in the case of pulmonary aspergillosis or Fusarium.\textsuperscript{40}

On the other hand, in combination with the observation of autoimmune IgG4 antibodies in EPF serum, another possible explanation of the high serum IgE levels in patients with EPF is isotype switching between IgG4 and IgE. In experimental models, (i.e., Brown Norway rats) mercury may induce an autoimmune syndrome in genetically sensitive strains.\textsuperscript{41} The syndrome is characterized by the deposit of linear IgG along the glomerular basement membrane, antinuclear antibodies, high serum levels of IgE, IgG1 and IgG4, and dermatitis.\textsuperscript{41} The mechanisms by which mercury generates this syndrome are as yet unknown, but similar changes also appear in animals treated with D-penicillamine or gold salts.\textsuperscript{42} Moreover, PF can also be triggered by D-penicillamine.\textsuperscript{43} Thus, a combination of several factors may be necessary to break self tolerance and result in xenobiotic-induced autoimmunity. Such factors likely compromise endogenous components such as T-cell receptors, regulatory T-cells, and human leukocyte antigen, but currently these ideas are speculative.

Endemic and epidemic diseases occurring in an acute or chronic manner, in a restricted, relatively well-defined geographic area, and arising as a result of autoimmune, degenerative and/or metabolic disorders, is not uncommon among people who may be genetically predisposed to react to different environmental factors. Examples of such diseases include pink disease (acrodynia), the increased autoimmunity associated with the Minamata Bay disaster and methyl mercury poisoning, generalized disorders of ectodermal tissue following prenatal exposure to polychlorinated biphenyls reported in Taiwan and Japan, the Apallic syndrome, Uric (Kashin-Beck) disease, eosinophilia-myalgia syndrome, hip disease seen in young Tunisia women, and desmosomal core protein in pemphigus foliaceus.\textsuperscript{44–45} In all these cases, a multidisciplinary approach for understanding the syndromes is necessary, since a multifactorial genetic and environmental cause appears probable.

ACKNOWLEDGMENTS

This work was supported by grants from the Embassy of Japan in Columbia, the University of Antioquia, Mineros de Antioquia S.A., and Lund University. We would like to extend our gratitude to the people of El Bagre and to all public and private Colombian companies that supported our program. We also want to thank Miss Olga Lucia Giraldo at the Department of Toxicology (U. de A.) for performing mercury detection.

REFERENCES