Profile of *Trypanosoma cruzi* Reactivity in a Population at High Risk for Endemic Pemphigus Folieus (Fogo Selvagem)


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Abstract. Fogo Selvagem (FS) is an autoimmune bullous disease with pathogenic IgG autoantibodies recognizing desmoglein 1 (Dsg1), a desmosomal glycoprotein. In certain settlements of Brazil, a high prevalence of FS (3%) is reported, suggesting environmental factors as triggers of the autoimmune response. Healthy individuals from endemic areas recognize nonpathogenic epitopes of Dsg1, and exposure to hematophagous insects is a risk factor for FS. Fogo selvagem and Chagas disease share some geographic sites, and anti-Dsg1 has been detected in Chagas patients. Indeterminate Chagas disease was identified in a Brazilian Amerindian population of high risk for FS. In counterpart, none of the FS patients living in the same geographic region showed reactivity against *Trypanosoma cruzi*. The profile of anti-Dsg1 antibodies showed positive results in 15 of 40 FS sera and in 33 of 150 sera from healthy individuals from endemic FS sites, and no cross-reactivity between Chagas disease and FS was observed.

INTRODUCTION

Pemphigus folieus (PF) is an autoimmune bullous dermatosis driven by immunoglobulin G (IgG) autoantibodies that recognize glycoproteins involved in epidermal adhesion. The clinical expression of the autoimmune process is blister formation, consequent to epidermal detachment (acantholysis). These autoantibodies bind to the extracellular domains of desmoglein 1 (Dsg1), a cadherin located in the desmosomal core of the keratinocyte surface.1-4

There are two main forms of PF: the classic one, with universal distribution, and the endemic form, also known as Fogo Selvagem (FS), prevalent in certain regions of Brazil and other Latin American countries.5,6 Main differences between the classic and the endemic presentation include peculiar epidemiological features, which are unique to FS, such as the presence of familial cases, involvement of children and young adults, and specific endemic settlements.6

The peak of FS in Brazil occurred in the first half of the 20th century. Aranha-Campos reported 604 cases through 1880 to 1940, where 26.5% were blood related7: a decline of 0.83 cases/10,000 inhabitants.8 Frequency of 30.7 FS cases/year through 1990 to 1999 in the State of Mato Grosso do Sul was detected.9 Endemic sites of PF were also found in other countries such as Colombia, Venezuela, Paraguay, and Peru.10-14

Fogo Selvagem has a complex pathogenesis, which includes genetic, immunological, and environmental factors. A Brazilian Amerindian Terena reservation, located at Limão Verde, Aquidauana, State of Mato Grosso do Sul (MS), with a high prevalence of FS (3%), has been closely followed up, once its main features includes a geographic, limited distribution of FS cases, that exhibit familial and temporal clustering.5,15-17

The immune response in FS is characterized by pathogenic IgG4 auto-antibodies that are driven to the extracellular 1 and 2 domains of Dsg1 (EC1-2).18 Interestingly, 55% of healthy individuals living in endemic FS areas generate anti-Dsg1 antibodies that recognize the extracellular 5 domain of Dsg1 (EC-5), a nonpathogenic epitope of the molecule. In those genetic predisposed individuals, intra-molecular spreading may occur, leading to an EC1-2-oriented IgG4 response, and therefore precipitating FS onset.6,18 There is also evidence of other immunoglobulin classes in FS pathogenesis: circulating IgM autoantibodies directed against Dsg1 are found in FS patients and in healthy individuals living in endemic areas, indicating a role as serological markers for the disease;19 moreover, an IgE-based immune response to Dsg1 was detected in the sera of 81% of FS patients.20 These findings lead to the hypothesis of continuous exposure to an environmental antigen that may share epitopes to Dsg1, and become a strong stimulus to nonpathogenic anti-Dsg1 IgM and IgG production in areas at high risk for FS.20

The genetic influence on FS is characterized by a positive association with the human leukocyte antigen alleles HLA-DRB1-0404, -1402 or -1406, with a relative risk of 14. A sequence of eight amino acids (LLEQRRAA) at the positions 67–74 in the third hypervariable domain of the DRB1 gene is shared by these alleles, conferring susceptibility to the disease.21,22

In genetically predisposed individuals, there may be triggers that initiate the immune response in FS through an antigen mimicry process.16 It is hypothesized that a break of immune tolerance follows exposure to some environmental factor(s) that include hematophagous insect bites, as reported elsewhere.16,23,24 The potential role of black fly triggering the autoimmune response in FS is supported by two main studies: exposure to simulid bites as a risk factor for FS (4.7 odds ratio),23 and the predominance of a certain black fly species (*Simulium nigrinum*) in endemic areas, when
compared with non-endemic areas of the Brazilian coast. Additional data on the environment triggers is suggested by Aoki and others, who reported a high frequency of black fly (87%), kissing bugs (67%), and bed bugs (60%) bites in FS patients, and showed that precarious living conditions represented a significant risk factor for FS. The sialotranscriptome of S. nigrum, the most common black fly species in endemic FS areas has been isolated, comprising over 70 distinct genes within over 30 protein families, offers an infinite source for testing pemphigus patients.

Some geographic areas of Brazilian vector-mediated tropical diseases, such as cutaneous leishmaniasis and Chagas disease, coincide with those endemic areas of FS. A previous work from our group evaluated the prevalence of anti-desmoglein 1 antibody in patients with cutaneous leishmaniasis, onchocerciasis, and Chagas disease, detecting a high prevalence of circulating autoantibodies directed against the nonpathogenic extracellular domain 5 of Dsg1 in Chagas disease (58%), leishmaniasis (43%), and onchocerciasis (81%).

Chagas disease is one of the major causes of cardiac chronic disease in Latin America, and is endemic in many regions of Brazil. However, in the State of Mato Grosso do Sul (MS), it is not considered an endemic disease because of the measures adopted for the vector’s control. Triatoma infestans, the main vector for Chagas disease in Brazil, has a low density in MS. Triatoma infestans was found among different regions in MS in the end of the 20th century (1980–2000), but it has been seldom detected in the last decade. On the other hand, different Triatominae species such as Triatoma sordida, Panstrongylus geniculatus, and Rhodnius neglectus with infection rates by Trypanosoma cruzi varying from 0.1% to 3.2% have been reported in this geographic region.

Information about the reactivity against T. cruzi of individuals from endemic areas of FS in the State of Mato Grosso do Sul is scarce. Therefore, this study aimed to characterize the immune response to T. cruzi in a population at high risk for FS.

MATERIALS AND METHODS

Geographic location of the endemic site. Limao Verde (LV) reservation is located 25 km Northeast of Aquidauana, and 160 km West of Campo Grande, the capital of the State of Mato Grosso do Sul (55°41′07″W, 20°19′00″S), as described elsewhere. It comprises a total of 1,712 hectares, with two well-defined geographical regions, LV and Corrego Seco. In September 2011, 1,349 people among 295 families inhabited LV reservation. The overall prevalence of FS in this endemic site was 3% in previous reports. Most of the houses have dirt floors, adobe walls, thatched roofs, poorly fitted or nonexistent doors, and no indoor plumbing or electricity. Poor toilet facilities are a common feature. The majority of individuals sleep on a raised platform covered by a variety of bedding material.

Study design. We included 30 FS patients from the LV Terena Reservation, MS, a FS focus where a clinical and immunological surveillance has been started since 1993 and 150 healthy individuals (selected at random from a total population of 1,349 inhabitants of LV) from September 2008 to September 2011. Blood samples were obtained by venipuncture and frozen at −70°C. All FS patients fulfilled clinical, histological, and immunofluorescence criteria for PF.

All participants were informed about the study and signed an informed consent, approved by the Ethics Committee (CAPPesq) from our institution.

Serologic assays. The collected samples of the selected individuals (FS patients and controls from LV) were analyzed for serological response against T. cruzi epimastigotes (Biomérieux, Marcy l’Etoile, France), using enzyme-linked immunosorbent assay (ELISA) and indirect immunofluorescence (IIF) with IgM and IgG (Biocientifica SA, Buenos Aires, Argentina) following standard procedures, according to manufacturers’ instructions as described elsewhere.

Immunoblotting with trypomastigote excreted-secreted antigens (TESA blot), an assay used as a confirmatory test for Chagas disease was performed in the five IIF positive sera to rule out cross-reactivity with leishmaniasis, and is briefly described as follows:

The TESA from the Y strain of T. cruzi were obtained as previously described. Briefly, the supernatants of LLC-MK2 cell cultures (in serum-free medium or with 2% fetal calf serum) infected with T. cruzi were collected when the concentration of trypomastigotes reached about 10–20 × 10^6/mL. After being centrifuged at 1800 × g for 15 min at 4°C, the supernatant containing TESA was then resubmitted to a second centrifugation (7000 × g for 5 min at 4°C) and used directly without any further treatment or stored at −80°C in small aliquots.

Proteins from TESA were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose sheets, and blocked with phosphate buffered saline (PBS) containing 5% fat-free milk for 1 h at room temperature. Membrane strips (5 mm) were incubated with human sera (1:200) diluted in PBS with 1% milk for 2 h or overnight at room temperature, washed, and the bound antibodies were detected with horseradish peroxidase (HRP)-labeled anti-human IgG (Sigma, St. Louis, MO), diluted (1:4000). The color of detected bands was developed by addition of 0.1% hydrogen peroxide and 4-chloro-1-naphthol. Samples were considered positive when a large 150–160 kDa band and/or five bands between 130 and 200 kDa were observed.

The serological profile of all sera (total IgG) against a recombinant form of human Dsg1, containing the entire extracellular domain and a C-terminal His-tag, described elsewhere, was tested by ELISA. Briefly, the ectodomain of Dsg1-1 with a carboxy-terminal His-tag were produced in High Five insect cells by infection with the recombinant baculovirus stock of Dsg1 (UNC Immunodermatology Laboratory, Department of Dermatology, University of North Carolina at Chapel Hill, NC). To generate the deglycosylated Dsg1, tunicamycin (Sigma) was added to the culture medium (0.5 μg/mL) at the time of infection. The tunicamycin-treated (deglycosylated) and untreated (glycosylated) recombinant Dsg1 was purified by nickel affinity chromatography and used for ELISA assay. The ELISA plates were coated with 200 ng/well of purified Dsg1 at 4°C overnight. After washing with Tris-buffered saline containing 3.7 mM Ca^2+ and 0.05% Tween-20 (TBS/Ca^2+/T-20), the plate was blocked with 1% bovine serum albumin (BSA) in TBS/Ca^2+/T-20 at room temperature for 1 h. The plate was then incubated with duplicate 1:10 dilutions of serum samples for 1 h at room temperature. Following wash, the plate was incubated with a 1:1000 dilution of HRP-labeled anti-human IgG or with a 1:2000 dilution of HRP-conjugated mouse anti-human IgG (Zymed, San Francisco, CA). Results were expressed as index
value units, and a cut-off value of 20 arbitrary units was used to separate positive from negative sera; values > 20 were considered positive.

RESULTS

Age and gender distribution. FS patients. There was a slight predominance of male patients (22 male: 18 female) and the median age was 32 years (ages ranging from 12 to 76 years). Close familial clustering was present in 34 of 40 (85%) of the patients, mostly parents/children or siblings. As for ethnic distribution, Terena Indians comprised the majority (32 of 40), and eight individuals were mestizos of Terena origin.

Non-FS individuals. In this clustered group of 150 individuals from LV, with a female predominance (89 female: 61 male), ages varied from 4 to 92 years, median age of 22.5 years; ethnic distribution revealed that 57% were Terena Indians and 43% mestizos.

Chagas disease assays. ELISA. The ELISA assays using T. cruzi epimastigotes for Chagas disease detected a negative response in 39 but one FS patient (cutoff 0.303, median 0.304) (Table 1).

The ELISA assays using T. cruzi epimastigotes for Chagas disease detected 5 of 150 non-FS patients from LV (cutoff 0.303, median 0.304) (Table 2).

IIF. None of the 40 FS sera showed reactivity against T. cruzi epimastigotes by IIF. The IgG antibodies that recognized T. cruzi epimastigotes were detected in 5 of 150 non-FS sera, titters varying from IIF 1:320 to > 1:640. Three of five individuals shared the same house, and were blood-related to FS patients. One of five individuals showed both IgM and IgG antibodies directed to T. cruzi.

TESA-blot. Five non-FS individuals confirmed indeterminate Chagas disease by positive results using immunoblotting with trypomastigote excreted-secreted antigens (Figure 1A and B).

Recombinant desmoglein 1 ELISA assay (total IgG). FS patients. Fifteen of 40 FS sera showed positive results by rDsg1 (Table 1), index values varying from 23 to 422 (mean: 133). The FS sera with negative rDsg1 results by ELISA corresponded to those in FS remission.

Non-FS individuals. Thirty-three healthy individuals out of 150 (22%) recognized rDsg1 by ELISA (Table 2), index values ranging from 20 to 227 (mean: 86). It is noteworthy to report that none of the non-FS individuals that recognized T. cruzi antigens showed positive ELISAs for rDsg1.

DISCUSSION

Endemic PF or FS represents a unique model of an autoimmune condition that may be triggered by environmental factors in genetic-prone individuals. The antigenic target is the extracellular portion of desmoglein 1, an adhesion molecule of the cadherin superfamily, which is recognized by pathogenic IgG, especially of the IgG4 isotype.6,22

The recognition of Dsg 1 epitopes is not restricted to patients with the disease, as previously reported.8 In this study, we detected 22% of reactivity against Dsg1 in non-FS individuals, indicating a possible environmental stimulus in the development of autoantibody formation. Some findings concerning the nonpathogenic response towards Dsg1 are relevant to reinforce the environmental hypothesis in FS, as follows: the lower prevalence of the IgG anti-Dsg1 response in normal subjects that live far away from the endemic sites (13%),32 the predominance of a certain black fly species, Simulium nigrimanum, in FS regions,24 an enhanced IgM or IgE anti-Dsg1 immune response in FS,19,20 and finally, the epidemiological data emphasizing the relevance of housing conditions and exposure to hematophagous insects, such as bedbugs and kissing bugs of patients with the disease.16

Individuals with parasitic diseases that are vector-mediated, such as onchocerciasis, cutaneous leishmaniasis, and Chagas disease profile and autoantibodies anti-desmoglein 1 tested by ELISA in non-Fogo Selvagem individuals from Limao Verde, MS

### Table 1

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<th>Chagas disease</th>
<th>Fogo Selvagem (rDsg1)</th>
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<th>Negative</th>
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* ELISA = enzyme-linked immunosorbent assay; rDsg1 = recombinant Desmoglein 1.

### Table 2

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<th>Non-FS individuals (rDsg1)</th>
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FS = Fogo Selvagem; rDsg1 = recombinant Desmoglein 1.

**Figure 1.** (A) Immunoblotting with trypomastigote excreted-secreted antigens (TESA blot) polypeptides recognized by IgG (lanes 1 to 5) from non-FS patients’ sera from Limao Verde (LV). (B) Lanes 1’ and 2’, positive and negative controls, respectively. Patients in lanes 1, 2, 3, 4, and 5 were classified as having indeterminate forms of Chagas disease, recognizing a 150- to 160-kDa band (chronic-phase antigen). Molecular mass markers are on the left. The molecular mass standards used were 205 kDa (rabbit muscle myosin); 116 kDa (Escherichia coli galactosidase); 97 kDa (rabbit muscle phosphorylase b); 66 kDa (bovine serum albumin).
disease, often possess circulating nonpathogenic anti-Dsg1 autoantibodies.\textsuperscript{17} In the pre-clinical phase of FS, there is an antigen-driven selection of anti-Dsg1 B cells,\textsuperscript{33} but the real source of this antigen remains to be determined.

Chagas disease is a major public health problem in many Latin America countries, but there are differences about its epidemiology in Amerindian populations living in these geographic sites.\textsuperscript{34} In native South American populations located in highlands (i.e., Bolivia, Argentina, Chile), records of Chagas disease date since the pre-Columbian era.\textsuperscript{34} Meanwhile, there is no report of the disease among lowland Amerindians or of Triatoma sp. living in their traditional houses.\textsuperscript{35}

Among the native Brazilian population, there is no evidence of Trypanosoma sp. in Xingu, Asurini Indians, Karitiana and Surui Indians.\textsuperscript{35} Coimbra\textsuperscript{36} performed a serological survey with the Xavante Indians from Mato Grosso, and found negative results in all 168 individuals tested for Chagas disease.

It has been hypothesized that Chagas disease is endemic in natives from the highlands because of features related to early domiciliation of triatomines and maintenance of the domestic cycle of T. cruzi.\textsuperscript{36,37} On the other hand, Amerindians in the lowland used to live in small settlements, with high village mobility and absence of domestic animals, similar to the conditions observed among native populations studied in Brazil.\textsuperscript{36}

The Terena population of LV reservation shows closer features with those populations of the highland Amerindians, i.e., animal domestication, raising indoor chickens, and low mobility. These conditions, when associated with the house with thatched roofs or adobe walls (Figure 2) provide an adequate environment to the domiciliation process of many species of triatomines. Our entomologist (DPE) performed an entomologic survey in LV, and detected four predominant Reduviidae species: Triatoma matogrossensis, Triatoma sordida, Rhodnius prolixus, and Panstrongylus geniculatus. Rates of natural infection with T. cruzi have been not yet recorded for those species (Eaton and others, unpublished data). These data are corroborated by previous performed studies on the profile of Reduviidae in a domestic environment in the State of Mato Grosso do Sul.\textsuperscript{26}

Although there were reports of the presence of triatomine inside the houses, and frequent exposure to kissing bugs in previous studies performed in the LV reservation, no Chagas disease has been so far detected in this endemic FS site, which has been followed up since 1993.\textsuperscript{15,16} By date, we did not find cross-reactivity with desmoglein 1 among T. cruzi positive sera in our samples, suggesting no relationship between T. cruzi and FS. In Brazil, there is a single report on IgG reactivity (38%) against trypomastigote forms of T. cruzi in eight sera from PF patients by IIF. However, there was no further information about those PF patients, and relevant data such as geographic location and demographic characteristics of these individuals were not available.\textsuperscript{38}

The mechanisms involved in anti-Dsg1 autoantibodies formation in patients with Chagas disease remain unknown. One of the possibilities includes compounds of hematophagous insect saliva inducing an immune response against Dsg1.\textsuperscript{17} It is interesting to note that antibodies of the IgG4 subclass directed against Triatoma infestans salivary gland proteins are produced by individuals living in triatomine-infested areas.\textsuperscript{39} Further studies are necessary to improve our understanding of such mechanisms; Assumpção and others\textsuperscript{40} recently described sialotranscriptome of T. matogrossensis from high-risk areas for FS, which may facilitate the identification of antigens with potential role for triggering FS, as well as development of biomarkers for low-level infestation of triatomines.

Conventional methods such as ELISA and IIF have been established for the serologic diagnosis of Chagas disease. In previous studies, ELISA sensitivity varied from 97.7% to 100%, and specificity, 93.3% to 100%; IIF showed sensitivity from 72% to 100% and specificity from 96% to 100%. Accuracy of both assays displays the best results in indeterminate and chronic stages, although there is frequent cross-reactivity, especially with leishmaniasis.\textsuperscript{41,42} Fortunately, TESA is a diagnostic method for Chagas disease, with 100% specificity, showing no cross-reaction for the 130- to 200-kDa antigen (acute-phase antigens) or the 150- to 160-kDa antigens (chronic-phase antigens).\textsuperscript{30} In our samples, TESA blot (Figure 1) showed bands that correspond to chronic-phase antigens, confirming the indeterminate stage for all five positive non-FS individuals from endemic areas of FS, previously tested by ELISA and IIF.

The description of reactivity against desmogleins 1 and 3 has been reported in patients from Tunisia, with visceral leishmaniasis (22%) and hidatisation (40%). In counterpart, no significant difference was found in PF patients and controls concerning the immune response against the above parasitic agents.\textsuperscript{43} Similarly, Brazilian patients with mucocutaneous leishmaniasis did show reactivity against Dsg1 in 43% of the cases; however, all FS patients from LV, except one, developed mucocutaneous leishmaniasis when tested by indirect immunofluorescence and ELISA (Diaz and others, unpublished data).

Pathophysiology of Chagas disease is complex and has been related to an autoimmune process. Immune response in T. cruzi chronic infection has features of delayed-type hypersensitivity with predominance of CD8\textsuperscript{+} over CD4\textsuperscript{+} T-cell subsets. Although cell-mediated immunity plays a central role in that process, humoral immunity may have participation through IgG directed against self-antigens such as neurons, sciatric nerve homogenates, and small nuclear ribonucleoproteins. Moreover, complement membrane attack complexes were identified in cardiac myocytes from Chagas disease patients.\textsuperscript{44}

Similar to FS, molecular mimicry appears as an important phenomenon in autoimmunity of Chagas disease. Trypanosoma cruzi antigens such as B13 protein, microsomal fraction,
sulfated glycolipids, and ribosomal protein have been described as molecules that may induce cross-reactivity with self-antigens found in human heart muscle (cardiac myosin, human ribosomal protein) or nervous tissue. Yet, anti-neuron autoantibodies found in Chagas disease may be linked to autonomic nervous system dysfunction in those patients. Moreover, T-cell clones sensitized to B13-protein in chronic Chagas disease with cardiomyopathy have been identified, showing multiple cross-reactive epitopes between T. cruzi B13 protein and human cardiac myosin heavy chain. 44

Our study revealed the occurrence of indeterminate Chagas disease in an Amerindian Terena population at high risk for FS in Brazil. Despite the absence of coexistence of the two conditions, clinical, epidemiological, and immune surveillance for FS and Chagas disease in this endemic area is mandatory, once both conditions share the same environmental milieu.

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