

Anti-Desmoglein-1 Antibodies in Onchocerciasis, Leishmaniasis and Chagas Disease Suggest a Possible Etiological Link to Fogo Selvagem

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Pemphigus foliaceus (PF) and the endemic form Fogo Selvagem (FS) are mediated by pathogenic antibodies to the EC1–2 domains of desmoglein-1. There is a preclinical phase with antibodies to only EC5. Based on geographic clustering of cases, FS is thought to have an, as yet unidentified, environmental trigger. In this study we have searched for anti-desmoglein-1 antibodies in sera from parasitic (leishmaniasis, Chagas, and onchocerciasis), and infectious diseases (leprosy and South American (SA) blastomycosis), which are prevalent in the same geographic regions of Brazil as FS. A specific and sensitive desmoglein-1 ELISA detected antibodies in 34 of 41 onchocerciasis (83%), 38 of 88 leishmaniasis (43%), 18 of 31 Chagas disease (58%), 7 of 28 SA blastomycosis (25%), and 14 of 83 leprosy sera (17%). These sera recognized epitopes restricted to the EC5 domain. These findings identify several etiological factors for FS. It is hypothesized that a component of insect vector saliva, rather than the parasite itself may trigger an antibody response to EC-5. In persons with the known HLA susceptibility alleles and living in endemic areas, a response to the EC1–2 domains may subsequently develop by epitope spreading with associated clinical signs of FS.

Key words: autoantibody/chagas/desmogleins/etiology/fogo selvagem/leishmaniasis/onchocerciasis/pemphigus
J Invest Dermatol 123:1045–1051, 2004

Both endemic (fogo selvagem (FS)) and non-endemic pemphigus foliaceus (PF) are characterized by subcorneal epidermal blisters and autoantibodies against desmoglein-1 (Dsg1) (Stanley *et al*, 1986; Diaz *et al*, 1989a). These autoantibodies are predominantly of the IgG4 subclass and pathogenic as demonstrated by passive transfer studies in neonatal mice (Roscoe *et al*, 1985; Rock *et al*, 1989). FS patients usually live in impoverished rural areas of certain states of Brazil where the disease is endemic (Paes-Leme, 1903; Vieira, 1937). Endemic PF has also been described in Colombia and Tunisia (Robledo *et al*, 1988; Morini *et al*, 1993). The disease exhibits a strong association with the HLA-DRB1*0404, 1402, or 1406 alleles ($p < 0.005$, RR: 14) (Moraes *et al*, 1997) and affects people of many races and ethnic backgrounds who live in endemic areas. Strikingly, the prevalence of FS in some states, e.g. São Paulo, has decreased dramatically in recent years (Diaz *et al*, 1989b). Additionally, a hospital case-control study previously showed that exposure to *simuliid* bites was 4.7 times more frequent in individuals developing FS than in those not ex-

posed to bites (Lombardi *et al*, 1992). It is thought that a local environmental agent or agents acting on genetically predisposed individuals triggers a pathogenic anti-Dsg1 antibody response that leads to FS.

We have identified a new focus of FS in the Amerindian Terena reservation of Limão Verde, Brazil, where the prevalence of the disease is 3.4% (Hans-Filho *et al*, 1996). We have reported previously that 30 of 31 patients along with 55% of normal subjects ($n = 93$) living in this reservation possessed anti-Dsg1 antibodies (Warren *et al*, 2000). A case-control study conducted in this reservation suggested that the presence of hematophagous insects in patient homes might be a relevant risk factor for FS (Aoki *et al*, 2004). Moreover, *Simulium nigricornum* (Eaton *et al*, 1998), *Cimex hemipterus*, *Triatoma matogrossensis*, and *T. sordida* have been identified in this reservation (D. Eaton *et al*, unpublished data). Neither *Trypanosoma cruzi* nor filarial parasites were identified by microscopic examination of these arthropods. Interestingly, although cutaneous leishmaniasis is prevalent in Limão Verde, Chagas disease and onchocerciasis are not, despite the presence of the arthropod vectors in this settlement.

Based on these observations, we hypothesized that an antigen(s) from blood feeding insects (*simuliids*, *phlebotomids*)

Abbreviations: Dsg, desmoglein; FS, fogo selvagem; PF, pemphigus foliaceus; SA, South American

mines, or *triatomines*) might trigger antibody formation in humans resulting in cross-reactive antibodies to epidermal Dsg1. Testing for disease-specific and cross-reactive antibodies against arthropod antigen(s) among the inhabitants of Limao Verde is problematic because not only FS patients, but also a significant number of normal individuals of this community possess anti-Dsg1 antibodies (Warren *et al*, 2000). To overcome this difficulty, we have searched for anti-Dsg1 antibodies in patients with tropical parasitic diseases where hematophagous vectors, found in Limao Verde, are involved in the transmission of illness, i.e. onchocerciasis (black flies), leishmaniasis (sand flies), and Chagas disease (kissing bugs). The Dsg1 reactivity of this group was compared with patients with infectious diseases (in which insect vectors are not involved) such as leprosy and South American (SA) blastomycosis, and a group of normal controls from Brazil and USA.

Results

The sera of patients with onchocerciasis, leishmaniasis, and Chagas disease recognize Dsg1 and its EC5 domain All FS/PF sera tested were positive (100% of 40) whereas all normal human sera from the USA were negative (100% of 57) by Dsg1-ELISA (Fig 1). This figure shows that the groups with the greater percentage of positive sera, aside from the FS/non-endemic PF group, were from patients with parasitic diseases such as onchocerciasis (83% of 41 tested), Chagas disease (58% of 31 tested), and leishmaniasis (43% of the 88 tested). Interestingly, the leishmaniasis groups from Brazil (73% of the 30 tested), Peru (31% of the 36 tested), and other parts of the world (36% of the 22 tested) were significantly different ($p = 0.0015$) (Table I).

In the infectious disease group, we found seven of the 28 SA blastomycosis sera (25%) and 14 of the 83 leprosy sera (17%) tested also reacted with Dsg1. Leprosy sera from Brazil exhibited a higher percentage of positive samples (24% of 37 samples) than samples from Mexico (12.1% of 33 samples) or other parts of the world (8% of 13 samples), but these differences were not statistically significant ($p = 0.3$) (Table I). The prevalence of antibodies directed against Dsg1 in sera from the onchocerciasis, leishmaniasis, and Chagas groups was significantly higher ($p < 0.001$) than the estimated true prevalence in this population of 20% previously reported by Warren *et al* (2000). In contrast, the sera from the SA Blastomycosis and leprosy group did not show a significant increase in prevalence above this estimated true prevalence.

We used immunoprecipitation as a second assay to detect the presence of anti-Dsg1 antibodies in the sera of onchocerciasis, leishmaniasis, and Chagas disease. The sera of these patients were tested using the full-length ectodomain of Dsg1. Dsg1 was detected using a monoclonal antibody against histidine residues that had been engineered into the recombinant protein. The results are summarized in Table II. Representative results of the immunoprecipitation studies depicted in Fig 2 show that 90% of the onchocerciasis sera ($n = 42$), 80% of the leishmaniasis sera ($n = 89$), and 55% of the Chagas sera ($n = 36$) were capable of immunoprecipitating Dsg1. Ninety-five percent of the USA normal human sera tested ($n = 39$) yielded negative results. There were two samples in this group that yielded a weakly positive reaction. Six bullous pemphigoid sera produced negative results.

For epitope-mapping studies, we selected sera from onchocerciasis ($n = 15$), leishmaniasis ($n = 11$), and Chagas ($n = 6$) that produced distinct positive reactions with the

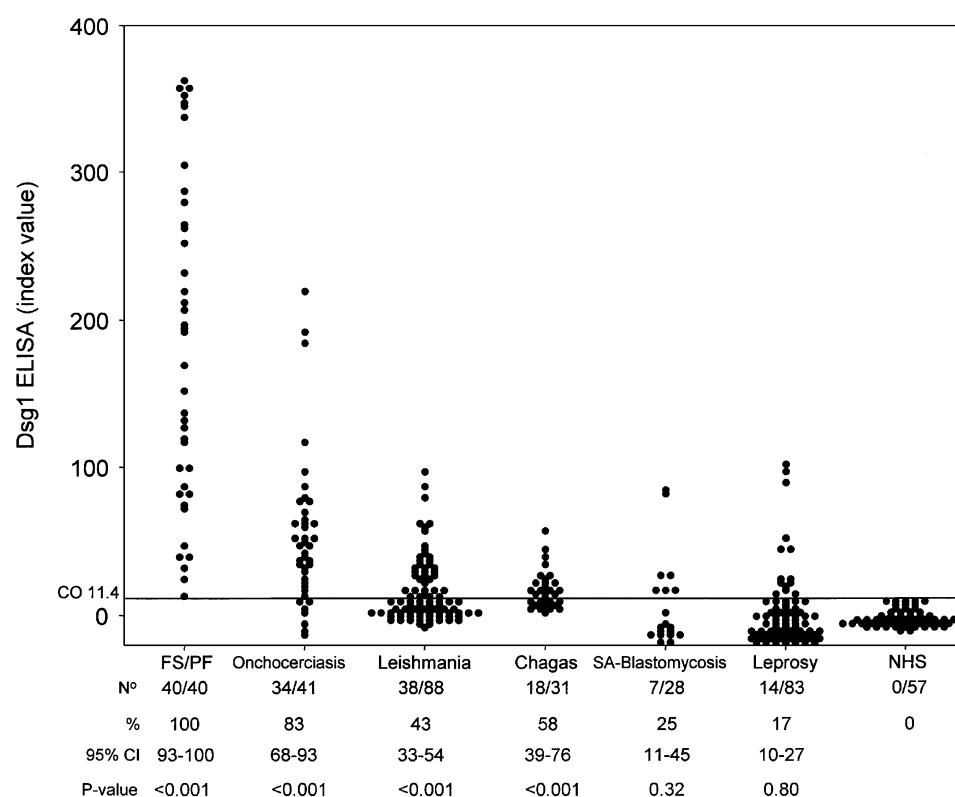


Figure 1

Desmoglein (Dsg)1-ELISA. A significant number of sera from patients with onchocerciasis, leishmaniasis, and Chagas possess anti-Dsg1 antibodies. The following data are given: number of positive/total (No.), % of positive sera, 95% confidence interval (CI), and the p-value. The p-value is the probability of observing prevalence similar to that actually observed, or higher, if the true prevalence were actually 20%. FS, Fogo Selvagem; PF, non-endemic pemphigus foliaceus; SA, South American; NHS, normal human serum from USA; CO, cut-off value.

Table I. Desmoglein-1 (Dsg1) ELISA results in leishmaniasis and leprosy sera from patients of different countries

	Leishmaniasis ^a	Leprosy ^b
Brazil	22/30 (73%)	9/37 (24%)
Peru	11/36 (31%)	NA
México	NA	4/33 (12%)
Other parts of the world ^c	8/22 (36%)	1/13 (8%)

^aLeishmania groups from Brazil, Peru, and other parts of the world were significantly different, $p=0.0015$.

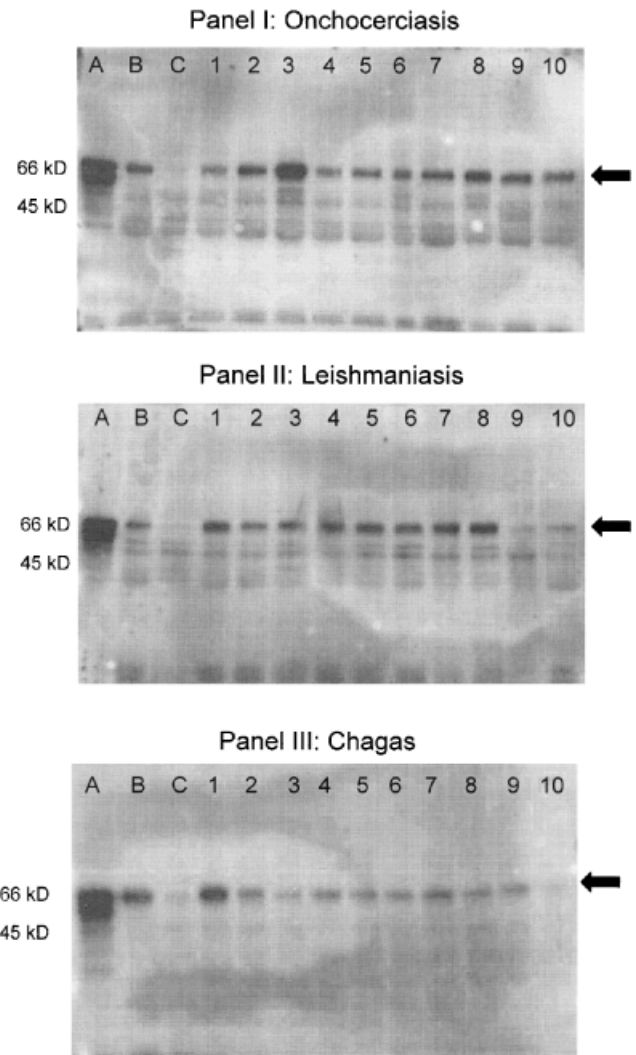
^bLeprosy groups from Brazil, Mexico, and other parts of the world were not significantly different, $p=0.3$; NA, not available.

^cCDC samples.

full-length Dsg1 ectodomain by immunoprecipitation assays. Four recombinant proteins were used, which included the full-length ectodomain of Dsg1, the EC1-4 domains, the EC5 domain, and the full-length ectodomain of Dsg3 (see Fig 3). Figure 4 shows representative examples of these studies. All onchocerciasis ($n=15$), leishmaniasis ($n=11$), and Chagas ($n=6$) sera recognized the EC5 domain of Dsg1. Six onchocerciasis sera and three leishmaniasis sera weakly recognized the EC4 domain of Dsg1. This group of sera reacted weakly with the EC1-4 construct, but did not react with the EC1-3 construct (data not shown). None of the sera tested reacted with the full-length Dsg3 ectodomain (the backbone molecule used to produce these recombinant Dsg1 hybrid proteins).

Reactivity of FS and control sera with trypanosoma, leishmania, and filarial antigens The sera of patients with FS ($n=20$), normal controls from Limao Verde (with and without anti-Dsg1 antibodies) ($n=10$), and non-endemic PF from the USA ($n=5$) were tested at Centers for Disease Control and Prevention (CDC) for antibodies against *T. cruzi* and *leishmania* by indirect immunofluorescence techniques. None of these sera reacted with the parasite antigens. Similarly, a group of 20 sera from FS patients tested by indirect immunofluorescence and ELISA techniques at the University of Sao Paulo, Brazil produced negative results using *T. cruzi* and *leishmania* antigens.

Fifty-four percent of sera from inhabitants of Limao Verde ($n=30$) possessed antibodies against a crude extract of filarial antigens (values above 13.5 μg per mL, which represents 3 SD above the mean of uninfected normal individuals); however, the prevalence of anti-filarial antibodies in the FS group (nine of 18) and the Limao Verde normal control group (six of ten) was similar. Moreover, anti-filarial antibodies in these sera were independent of anti-Dsg1

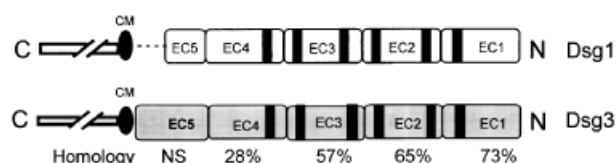
**Figure 2**

Detection of anti-desmoglein (Dsg1) antibodies by immunoprecipitation assays. Each panel contains immunoprecipitates produced by a well-characterized strong fogo selvagem (FS) serum (1:400 dilution) (lane A), a weak FS serum (1:100 dilution) (lane B), and a USA normal human serum (1:100 dilution) (lane C). Lanes 1-10 of each panel contain serum samples (1:100 dilution) from onchocerciasis, leishmaniasis, and Chagas disease, respectively. The molecular weight is shown on the left of each panel. An arrow on the right of each panel shows the location of Dsg1. FS serum A (lane A) shows strong positive staining in all panels. FS serum B (lane B) shows good staining of Dsg1. Normal human serum C (lane C) shows no reactivity. Serum #1 of panel I (onchocerciasis) shows weak positive staining, whereas sera 2-10 show good positive staining of Dsg1. Sera #1-8 of panel II (leishmaniasis) show good positive staining; whereas, sera #9 and 10 show negative staining. Serum #1 of panel III (Chagas) shows good positive staining; sera 2-9 show weak positive staining, whereas serum #10 is negative.

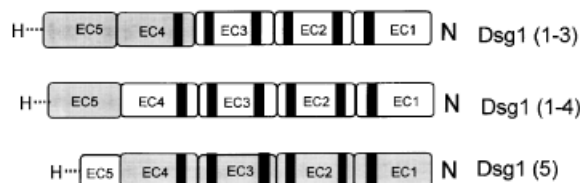
Table II. Summary of immunoprecipitation results for anti-desmoglein-1 (Dsg1) antibodies

Reactions	Onchocerciasis		Leishmaniasis		Chagas		Normal sera (USA)		Bullous Pemphigoid	
	Number (n = 42)	%	Number (n = 89)	%	Number (n = 36)	%	Number (n = 39)	%	Number (n = 6)	%
Positive	32	76	51	57	7	19	0	0	0	0
Weak positive	6	14	20	23	13	36	2	5	0	0
Negative	4	10	18	20	16	44	37	95	6	100

Full-length ectodomains of desmoglein 1 and desmoglein 3



Desmoglein 1 and desmoglein 3 hybrid proteins

**Figure 3**

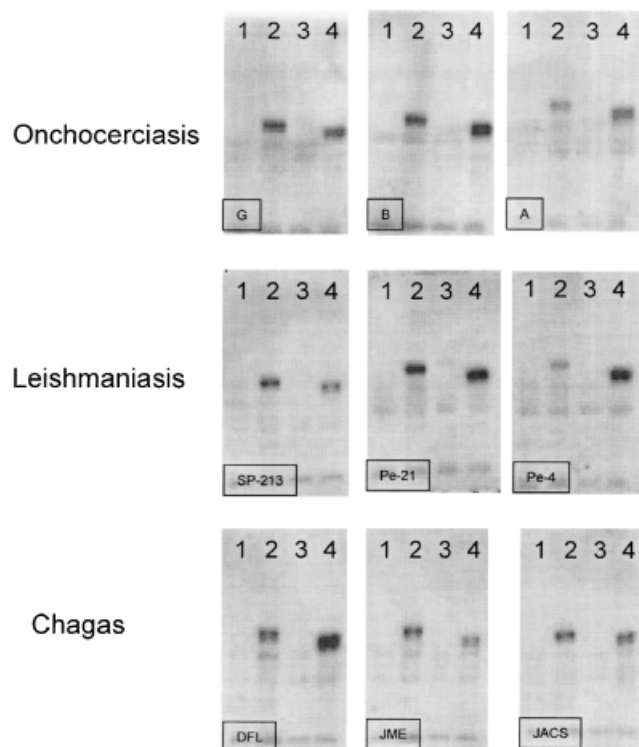
Molecular structures of desmoglein (Dsg)1, Dsg3, and chimeric antigens. The top panel shows the molecular structures of Dsg1 and Dsg3. The cell membrane (labeled CM) and the N and C terminal of each molecule are as depicted. Each ectodomain of the molecule contains five domains labeled EC1, EC2, EC3, EC4, and EC5. The degree of sequence identity between each domain of Dsg1 (white) and Dsg3 (gray) are indicated. The EC5 domains of these molecules exhibit no significant homology (NS). The black strips represent Ca^{2+} binding motifs. The bottom panel shows the domain structure of the three chimeras of Dsg1 and Dsg3 used in this investigation. The Dsg1 (1-3) chimera spans domains 1-3 of the Dsg1 molecule (shown in white); the Dsg1 (1-4) chimera spans domains 1-4 of Dsg1 and chimera Dsg1 (5) contains domain 5 of Dsg1 only. A six-histidine tag (H) was attached to the C-terminus of the recombinant proteins.

antibodies (data not shown). Five sera from non-endemic PF showed low titers of anti-filarial antibodies (less than 6.3 μg per mL).

Discussion

This investigation demonstrated that a significant number of patients with parasitic diseases such as onchocerciasis, leishmaniasis, and Chagas disease possess anti-Dsg1 antibodies in their sera when tested against the full-length ectodomain of Dsg1 using both highly specific and sensitive ELISA and immunoprecipitation assays. Importantly, none of these patients showed evidence of skin disease suggestive of FS. The control sera, which include normal human donors from the USA and patients with bullous pemphigoid, yielded negative tests for anti-Dsg1 antibodies, demonstrating the specificity of our assays.

The results shown in Fig 1 and Table II demonstrate that over 80% of the onchocerciasis sera possess anti-Dsg1 antibodies by these two assays (83% of 41 tested by ELISA and 91% of 42 tested by immunoprecipitation). Similarly, over 50% of the Chagas sera exhibited anti-Dsg1 antibodies (58% of 31 tested by ELISA and 55% of 36 tested by immunoprecipitation). In the leishmaniasis group the ELISA assay detected 43% of positive sera in a large cluster of sera ($n=88$) from Brazil, Peru, and CDC; whereas, by immunoprecipitation, 80% of the same sera showed positive results. The ELISA results of the leishmaniasis group showed substantial differences in the number of positive sera in donors from Brazil (73% of 30 tested) as compared

**Figure 4**

Epitope mapping studies of anti-desmoglein (Dsg)1 antibodies from representative onchocerciasis, leishmaniasis, and Chagas sera. The full-length ectodomains of Dsg3 (lane 1) and Dsg1 (lane 2), the hybrid proteins containing the EC1-4 domain (lane 3) and the EC5 (lane 4) domain of Dsg1 were used in immunoprecipitation procedures. All sera recognized the EC5 domain of Dsg1. The 15 sera from onchocerciasis, 11 from leishmaniasis, and six from Chagas disease did not react with Dsg3. Additionally, six onchocerciasis sera bound weakly the EC4 domain of Dsg1 (data not shown).

with donors from Peru (31% of 36 tested) and other parts of the world (36% of 22 sera tested), ($p=0.0015$) (Table I).

Other groups that showed lower reactivity with Dsg1 by ELISA comprised patients with tropical infectious diseases such as SA blastomycosis (25% of 28) and leprosy (17% of 83). The differences in percent positive tests between Brazilian leprosy patients (24% of 37), patients from Mexico (12% of 33), or other parts of the world (8% of 13) were not statistically significant ($p=0.3$) (Table I). Since FS is endemic to the same Brazilian regions from where the leishmaniasis, SA blastomycosis, and leprosy sera were collected, these intriguing results suggest a shared environmental antigen(s) triggering anti-Dsg1 antibody production in these patients.

If the background prevalence of anti-Dsg1 antibodies in endemic areas of FS is 20% (Warren, 2000) the high prevalence of these antibodies in patients with parasitic diseases (onchocerciasis, leishmaniasis, and Chagas) is significant ($p<0.001$).

Epitope mapping studies using a representative number of positive sera of each group (15 onchocerciasis, 11 leishmaniasis, and six Chagas) and recombinant fragments of the Dsg1 ectodomain and the full-length Dsg3 ectodomain revealed that all samples tested from onchocerciasis, leishmaniasis, and Chagas disease recognized the EC5

domain of Dsg1 (Fig 4). Importantly, none of these sera recognized Dsg3, which is another indicator of the specificity of the immunoprecipitation assay in detecting anti-Dsg1 antibodies. Li *et al* (2003) have recently demonstrated that the EC5 domain is also recognized by sera from normal individuals from Limao Verde (exhibiting anti-Dsg1 antibodies) and FS patients during the preclinical stage of the disease. They hypothesized that this domain might contain the initial epitope(s) that trigger(s) the autoimmune response in FS.

The presence of anti-Dsg1 antibodies specific for the EC5 domain in patients with vector-mediated parasitic diseases implies immunization with an environmental EC5-cross-reactive antigen(s). The present studies suggest that these cross-reactive antigen(s) may originate from the insect vector(s) of onchocerciasis, leishmaniasis, and Chagas disease themselves. In addition to their own sensitizing products (e.g. saliva), however, these vectors carry parasites that cause the respective diseases. Importantly, FS sera did not recognize *trypanosoma* or *leishmania* antigens. Unexpectedly, we found that 50% of the sera from Limao Verde (patients and controls) recognized filarial antigens by an ELISA assay using crude filarial antigens; however, the prevalence of anti-filarial antibodies in sera from FS patients and controls was independent of the presence of anti-Dsg1 antibody titers by indirect immunofluorescence and/or ELISA. The low specificity of the assay (Lal and Ottesen, 1988) and the high prevalence of non-filarial intestinal parasites in the Limao Verde population (unpublished data) may explain these findings.

Another source(s) of sensitizing antigen(s) leading to anti-Dsg1 response in onchocerciasis, leishmaniasis, and Chagas disease might be the saliva of the hematophagous vector, which is known to induce antibody responses in humans (Barral *et al*, 2000). Interestingly, the genome of the fruit fly *Drosophila melanogaster* contains 18 cadherin family member proteins, which bear structural similarities to human Dsg1 (Hill *et al*, 2001) suggesting that hematophagous vectors may similarly possess numerous cadherin family members in their structural or secreted proteins. It is unclear whether the sensitizing antigen(s) is the same in all three vectors (*simuliids*, *phlebotomines*, or *triatomines*) or whether they represent different molecules sharing the same Dsg1 cross-reactive epitope. It is also important to consider that individuals living in these endemic regions are usually exposed simultaneously to all three arthropod bites. These individuals may develop the respective tropical disease (leishmaniasis, Chagas, onchocerciasis), but may additionally exhibit antibodies to the other vectors to which they were exposed. Therefore, each disease group may possess anti-Dsg1 antibodies produced in response to an antigen(s) from a single vector or from all three arthropods.

In conclusion, if arthropod bites trigger a non-pathogenic anti-Dsg1 antibody response in humans, this phenomenon would likely be common to other parts of the world where individuals are similarly exposed to these vectors. The rarity of FS and its unique epidemiological features, such as the clustering of cases in certain endemic regions of Brazil, require further explanation. Based on the present findings and recent studies by Li *et al*, we propose that an arthropod salivary antigen(s) triggers an early antibody response that

cross-reacts with the EC5 domain of Dsg1 by molecular mimicry (Oldstone, 1987). Since these anti-EC5 antibodies are non-pathogenic the majority of these individuals remain disease-free. Only a small minority of subjects, those that will develop FS, might extend their immune response by epitope spreading (Lehmann *et al*, 1993; Li *et al*, 2003) to include pathogenic antibodies against the EC1 and EC2 domains of Dsg1. Additionally, the autoimmune disease in these patients may be perpetuated by continuous exposure to self-Dsg1. The high prevalence of FS in Limao Verde may have resulted from inbreeding of the population resulting in a large number of genetically predisposed individuals who are repeatedly exposed to a sensitizing agent(s). Experimental confirmation of this hypothesis may have important implications in understanding human autoimmune disease.

Materials and Methods

Sources of sera for assaying anti-Dsg1 antibodies Several groups of sera were tested: (a) This set comprised samples obtained from patients with parasitic diseases where an insect vector transmits the parasite that causes the illness. This group included sera from patients with onchocerciasis (n=42), leishmaniasis (n=89), and Chagas disease (n=36). Twenty-nine sera from patients with leishmaniasis (23 cutaneous and six mucocutaneous) and 31 from Chagas disease were obtained from the Department of Dermatology of the University of Sao Paulo (USP), Brazil. These patients originated from the State of Sao Paulo and neighboring states and had no cutaneous signs of FS. The diagnosis of leishmaniasis and Chagas disease was confirmed by histological examination, indirect immunofluorescence, and ELISA assays. Other well-characterized sera from 25 patients with leishmaniasis from different continents and countries (Central America, n=10; Middle East and Asia, n=6; Europe, n=2; South America, n=4; USA, n=1; unknown, n=2) and five patients with Chagas disease were obtained from the Division of Parasitology, Centers for Disease Control and Prevention (CDC), Atlanta. Additionally, we obtained 35 serum samples from leishmaniasis patients (31 mucocutaneous form and four cutaneous) from the laboratory of Dr Antunez de Mayolo from Lima, Peru, and 42 sera from onchocerciasis patients (18 from Guatemala and 24 from Africa) from the laboratory of Dr Nutman, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda, Maryland.

(b) Another group of sera was obtained from patients with tropical infectious diseases such as SA blastomycosis (n=28) and leprosy (n=83). The 28 SA blastomycosis sera and 37 of the leprosy sera were also obtained from the Department of Dermatology USP, Brazil. Forty-six leprosy sera (33 from Mexican patients and 13 from other countries) were supplied by Dr Thomas Rea from the LAC-USC Medical Center, Los Angeles, California. The diagnosis of SA blastomycosis was established by clinical, histological, and mycological criteria.

(c) The third group contained sera from USA normal individuals (n=57). To assess the background prevalence of anti-Dsg1 antibodies in a normal Brazilian population, we use previously published ELISA data (Warren, 2000). Briefly, in this study we found that out of 279 normal individuals living outside of Limao Verde (high prevalence of FS), 54 possessed anti-Dsg1 antibodies (~20%). This 20% background prevalence of anti-Dsg1 antibodies, 18 of 96, has also been recently found in Parana, another Brazilian region where FS is endemic (Empinotti, unpublished data).

(d) The specificity of the immunological assays was also tested using sera from patients with bullous pemphigoid (n=6) from the USA containing antibodies against two antigens, BP230 and BP180 (Giudice and Diaz, 1996).

Other sera tested for antibodies against leishmania, trypanosoma, and filarial antigens We also sent well-characterized sera to the following laboratories: (a) CDC, for assaying antibodies against *T. cruzi* (Chagas disease parasite) and *leishmania* (leishmaniasis parasite) antigens: FS ($n=10$), non-endemic PF ($n=5$), and sera from normal individuals ($n=10$) from Limao Verde containing anti-Dsg1 antibodies (Warren *et al*, 2000). The sera were tested against *T. cruzi* (epimastigote stage from a Brazilian strain) and *leishmania* (promastigote stage, WHO-DD8 from India) by indirect immunofluorescence techniques. (b) Dr Nutman at the NIAID: for assaying antibodies against filarial antigens (onchocerciasis parasite): FS sera from Limao Verde ($n=20$), non-endemic PF from USA ($n=5$), normal donors from Limao Verde showing anti-Dsg1 antibodies ($n=5$), and normal donors from Limao Verde exhibiting negative anti-Dsg1 serology ($n=5$). Anti-filarial antibodies by an ELISA assay at the laboratory of NIAID using a PBS-soluble extract of *Brugia malayi* (Hamilton *et al*, 1981). (c) Finally, a set of 20 well-characterized sera from FS patients seen at the University of Sao Paulo were also tested for antibodies against antigens from *T. cruzi* and *leishmania* sp. by commercially available indirect immunofluorescence and ELISA techniques (Immunocruzi, Biolab, Hemobio, Embrabio and Instituto Adolfo Lutz from Sao Paulo, Brazil).

The sera were kept frozen at -20°C and transported from Brazil, Peru, CDC, and NIAID to our laboratory at UNC following CDC regulations. Each group of sera was anonymized by code following their arrival in our laboratory. All described studies had the approval of the IRB at University of North Carolina, and the study was conducted according to the Declaration of Helsinki Principles.

Construction and production of recombinant Dsg1, Dsg3, and chimeric antigens The desmosomal cadherins Dsg1 and Dsg3 are targets of pathogenic antibodies in patients with the two distinct autoimmune diseases non-endemic PF (and FS) and pemphigus vulgaris, respectively (Udey and Stanley, 1999). As shown in Fig 3 (*top*), the ectodomains of Dsg1 and Dsg3 have extensive sequence homology. We have constructed and expressed in the baculovirus system the entire extracellular domains of human Dsg1 and Dsg3 (Ding *et al*, 1997, 1999). Recently, we have engineered and expressed in baculovirus eight hybrid molecules of Dsg1 and Dsg3 (Li *et al*, 2003) in which the ectodomains of the two antigens were swapped as has been described by others (Netzer *et al*, 1999; Sekiguchi *et al*, 2001). Three of these hybrid molecules (EC1-3, EC1-4, and EC5) were chosen for epitope-mapping studies (Fig 3, *bottom*). All the recombinant proteins were histidine tagged at the C-terminal of the molecule.

ELISA assays We used a Dsg1-ELISA assay as previously reported (Warren *et al*, 2000; Arteaga *et al*, 2002) with the following modifications. Purified histidine-tagged recombinant Dsg1 was used to coat the nickel-NTA plates (Qiagen, Valencia, California) at 50 ng per well. Patient or normal subject sera were added to the coated plate in duplicate at a dilution of 1:200 in TBS- Ca^{2+} buffer, pH 7.2, containing 1% BSA and 0.05% Tween 20. The plate was then incubated with HRP-mouse monoclonal anti-human IgG (Zymed Lab, San Francisco, California) diluted to 1:1000 in TBS- Ca^{2+} pH 7.2/1% BSA/0.05% Tween 20. The same positive and negative controls were used in duplicate on each ELISA plate. Duplicate ELISA results were averaged. An ELISA index value was obtained as follows: [(test result–negative control)/(positive control–negative control) $\times$ 100]. A cut-off value was established as a value 2.5SD above the mean of the 57 normal donors from the USA. Several sera generated negative values after subtraction of background and correction for plate-to-plate variability, as their OD value was below the negative control.

Statistical analysis The confidence intervals and tests for binomial proportions were based on exact binomial calculations. The p -values in Fig 1 were based on testing the null hypothesis that the true prevalence of positives (background prevalence for people living in endemic areas of FS in Brazil, outside Limao Verde) is 20% (Warren, 2000) against the alternative that it is greater than 20%.

Other tests for proportions were two sided and based on Fisher's exact test.

Immunoprecipitation assays Recombinant Dsg1 (0.5 μg) was immunoprecipitated with each test serum (0.5 μL) using a protocol as previously reported (Li *et al*, 2003). Two sera from FS patients (A, B) and one from a USA normal human donor (C) were used throughout as controls. Serum A showed an indirect immunofluorescence titer of 1:640, serum B a titer of 1:20, and serum C was negative. The three control sera (A, B, and C) and 10 test sera were processed simultaneously in each experiment. Serum A was tested at a 1:400 dilution; whereas, serum B and normal human serum at a dilution of 1:100.

This work was supported in part by US Public Health Service Grants R01-AR30281, R01-AR32599, and T32 AR07369 awarded to Dr LA Diaz. We acknowledge the invaluable advice of Dr Jose M. C. Ribeiro, PhD from the Intramural Program of the National Institute of Allergy and Infectious Diseases (NIAID). Mr J. Kubofcik, from the Laboratories of Parasitic Diseases, NIAID, Bethesda, Maryland, performed the anti-filarial tests and Mr F. Steurer from CDC-Parasitology, provided leishmaniasis and Chagas sera as well as performed the test for Chagas and leishmaniasis. We also acknowledge the support of Dr L. M. Ito, Dr G. Tamai, Dr C. W. Maruta, Dr C. G. Santi, Dr M. Juvenale, and Ms B. J. Celeste, Mr A. M. Perigo, Ms L. M. Fukumori, and Dr M. A. Belez from the University of Sao Paulo, Brazil. Dr Thomas H. Rea from the LAC-USC Medical Center, Los Angeles, California supplied leprosy sera. DOI: 10.1111/j.0022-202X.2004.23438.x

Manuscript received February 6, 2004; revised June 1, 2004; accepted for publication June 7, 2004

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