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IgG Autoantibody Response against Keratinocyte Cadherins in Endemic Pemphigus Foliaceus (Fogo Selvagem)

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It is well established that autoantibodies against desmoglein 3 and desmoglein 1 (Dsg1) are relevant in the pathogenesis of pemphigus vulgaris and pemphigus foliaceus, including its endemic form fogo selvagem (FS). Isolated reports have shown that in certain patients with these diseases, autoantibodies against other desmosomal cadherins and E-cadherin may also be present. The goal of this investigation was to determine whether FS patients and normal individuals living in endemic areas possess autoantibodies against other desmosomal cadherins and E-cadherin. By testing a large number of FS and endemic control sera by ELISA, we found a consistent and specific autoantibody response against Dsg1 and other keratinocyte cadherins in these individuals, which is quite different from healthy individuals from the United States (US controls). Overall, the highest correlations among the autoantibody responses tested were in the endemic controls, followed by FS patients, and lowest in the US controls. These findings suggest that multiple, perhaps cross-reactive, keratinocyte cadherins are recognized by FS patients and endemic controls.

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INTRODUCTION

Both nonendemic pemphigus foliaceus (PF) and fogo selvagem (FS) show subcorneal epidermal blisters and pathogenic anti-desmoglein 1 (Dsg1) IgG autoantibodies (Beutner and Jordon, 1964; Beutner *et al.*, 1968; Roscoe *et al.*, 1985; Stanley *et al.*, 1986a, 1986b; Diaz *et al.*, 1989a). FS patients usually live in rural areas of certain states of Brazil where the prevalence of disease is higher than in urban communities (Diaz *et al.*, 1989b). The disease exhibits a strong association with the HLA-DRB1*0102, 0404, and 1402 alleles (Moraes *et al.*, 1997). It is thought that an environmental antigen(s) may trigger FS (Flores *et al.*, 2009).

One of the relevant target antigens in both PF and FS is the desmosomal cadherin Dsg1 (Stanley *et al.*, 1986a; Buxton *et al.*, 1993). Desmosomal cadherins are transmembrane glycoproteins that are critical components of the desmosome, one of the keratinocyte intercellular junctions (Desai *et al.*, 2009). They include the Dsgs and desmocollins (Dscs), each with well-characterized isoforms; four Dsgs (Dsg1–4) and three Dscs (Dsc1–3; Thomason *et al.*, 2010). Desmosomal cadherins are structurally and functionally similar to the classic E-cadherin, which is the molecular component of the adherens junction. Adherens junctions are structurally located in the interdesmosomal regions of the keratinocyte cell surface (Green *et al.*, 2010).

IgG antidesmosomal autoantibodies from PF and FS sera are routinely detected by indirect immunofluorescence, which have been used as a diagnostic test for these diseases for decades. The application of ELISA techniques using recombinant human Dsg1 has improved diagnostic accuracy (Amagai *et al.*, 1999), and has made it possible to test large numbers of individuals living in communities in which FS is endemic (Warren *et al.*, 2003; Qaqish *et al.*, 2009). ELISA studies have also identified IgG4 anti-Dsg1 autoantibodies as a serological predictor of FS (Warren *et al.*, 2003; Qaqish *et al.*, 2009). Moreover, anti-Dsg1 autoantibodies of the IgM (Diaz *et al.*, 2008) and IgE (Qian *et al.*, 2011) class have also been detected in the sera of a large number of FS patients, suggesting an ongoing environmental trigger. Although anti-Dsg1 IgG and IgG4 autoantibodies have been shown to be pathogenic in PF and FS (Rock *et al.*, 1989), there have been reports of autoantibody responses to other members of the

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Abbreviations: AUC, area under the curve; CI, confidence interval; Dsc, desmocollin; Dsg1, desmoglein 1; Dsg3, desmoglein 3; FS, fogo selvagem; OD, optical density; PF, pemphigus foliaceus; ROC, receiver-operating characteristic; TBS, Tris-buffered saline; T-20, Tween-20

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cadherin family of proteins in certain pemphigus phenotypes. Dsg3 is diagnostic and pathogenically relevant in pemphigus vulgaris (Amagai *et al*., 1991), whereas Dsc antigens may be relevant in IgA pemphigus (Duker *et al*., 2009) and certain forms of pemphigus vulgaris (Mao *et al*., 2010). Autoantibodies against Dsg4 (Nagasaka *et al*., 2004) and E-cadherin (Evangelista *et al*., 2008) have also been described in pemphigus vulgaris and PF, respectively.

It is accepted that the desmosome, specifically its structural component Dsg1, is the target of pathogenic IgG4 polyclonal autoantibodies generated by FS patients. The IgG response in FS is complex and may result from exposure to environmental antigens and/or to self-Dsg1. Moreover, the final pathogenic IgG4 response may evolve via phenomena such as cross-reactivity with other desmosomal cadherins and/or epitope spreading. The aim of this study, therefore, was to determine the IgG autoantibody profile for each of the eight keratinocyte cadherins in a large number of subjects from three groups, i.e., FS patients, healthy individuals from the United States (US controls), and healthy individuals living in an endemic area of FS (endemic controls). We find that a substantial number of FS patients and endemic controls possess autoantibodies not only to Dsg1 but to other keratinocyte cadherins as well. Some of these autoantibody responses show strong correlations in the FS and endemic control groups.

RESULTS

Index value distribution and comparison of eight antigen/antibody systems in FS patients and two healthy control groups

Sera of 101 FS patients, 106 US controls, and 106 endemic controls were tested for the presence of IgG autoantibodies to Dsg1, Dsg2, Dsg3, Dsg4, Dsc1, Dsc2, Dsc3, and E-cadherin by ELISA. The distribution of index values for each antigen/antibody system is depicted in a box plot format (Figure 1). Median index values are reported in Table 1. To evaluate for significant differences, the data were further analyzed using 24 pairwise comparisons (three groups of sera compared with each other for each of the eight antigen/antibody systems). A Bonferroni correction was used to adjust for multiple comparisons among the 24 tests; thus, for an overall α of 0.05, only *P*-values below $0.05/24=0.0021$ are considered significant (Table 1).

As expected, we find higher anti-Dsg1 index values in FS patients than in the US controls and endemic controls (median 108.79 vs. −22.83 and −9.26, respectively; $P<0.0001$). The differences are also significant when comparing index values of FS patients and the US controls in five of the seven remaining antigen/antibody systems (Dsg4 and Dsc2 were not significant). In addition, the differences between index values of FS patients and endemic controls are all significant, except for Dsc2 and E-cadherin responses ($P=0.4033$ and $P=0.2524$, respectively).

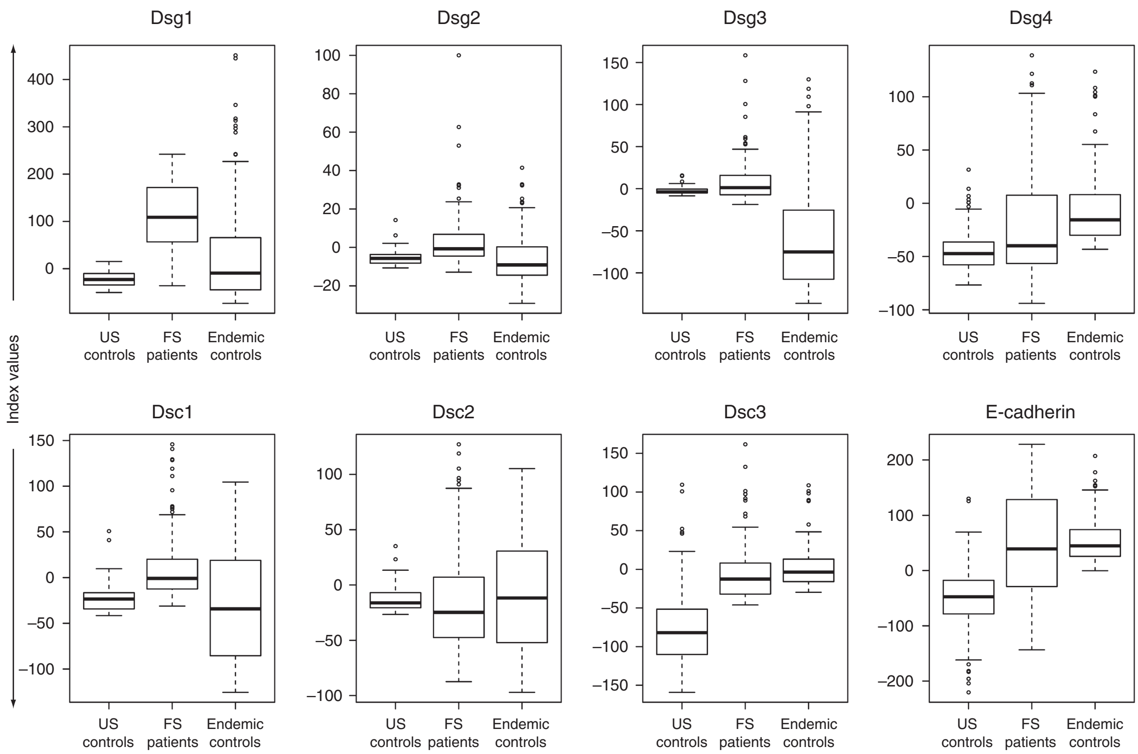

Figure 1. The distribution of index values among healthy individuals from the United States (US controls), fogo selvagem (FS) patients, and endemic controls. Dsg1 ELISA index values show the greatest separation of FS patients from both sets of controls ($P<0.0001$), but show no difference between the control groups ($P=0.0162$). Each box extends from the lower to the upper quartile (25–75%), with the median shown as a thick line within the box. The whiskers extend away from the box to the most extreme data point, which is no more than 1.5 times the length of the box. Data points beyond the whiskers are individually marked. Dsc, desmocollin; Dsg, desmoglein.

Table 1. Comparison of distributions of index values for the US controls, FS patients, and endemic controls[1]

	Index value medians			*P*-values for comparison of index value distributions		
	US controls	FS patients	Endemic controls	US controls vs. FS patients	US controls vs. endemic controls	FS patients vs. endemic controls
Dsg1	−22.83	108.79	−9.26	<0.0001*	0.0162	<0.0001*
Dsg2	−5.72	−0.73	−9.09	<0.0001*	0.015	<0.0001*
Dsg3	−3.78	1.21	−75.16	0.001*	<0.0001*	<0.0001*
Dsg4	−47.28	−39.93	−15.59	0.0088	<0.0001*	<0.0001*
Dsc1	−23.51	−0.86	−34.13	<0.0001*	0.1119	<0.0001*
Dsc2	−16.22	−24.65	−11.68	0.012	0.802	0.4033
Dsc3	−82.00	−12.62	−3.57	<0.0001*	<0.0001*	0.0007*
E-cadh	−47.47	39.11	44.67	<0.0001*	<0.0001*	0.2524

Abbreviations: Dsc, desmocollin; Dsg, desmoglein; E-cadh, E-cadherin; FS, fogo selvagam.
[1]The data from which these medians and *P*-values were derived are depicted in Figure 1. To adjust for multiple comparisons with an overall α of 0.05, only *P*-values below 0.05/24=0.0021 are considered statistically significant (marked by an asterisk (*)). The total number of subjects in each group is as follows: US controls (n=106), FS patients (n=101), and endemic controls (n=106).
Index value medians and *P*-values for all pairwise comparisons using the Wilcoxon two-sample test are shown.

Comparison of index values of the US controls and endemic controls reveals statistically significant differences for four antigen/antibody systems (Dsg3, Dsg4, Dsc3, and E-cadherin). There are three antigen/antibody systems in which the endemic controls have higher index values than the US controls, i.e., Dsg4 (median −15.59 vs. −47.28; <0.0001), Dsc3 (median −3.57 vs. −82.00; $P<0.0001$), and E-cadherin (median 44.67 vs. −47.47; $P<0.0001$), and one in which the index values of the US controls are higher than those in endemic controls, i.e., Dsg3 (median −3.78 vs. −75.16; $P<0.0001$). The differences in index values for the remaining antigen/antibody systems were not significant (Dsg1, $P=0.0162$; Dsg2, $P=0.0150$; Dsc1, $P=0.1119$; and Dsc2, $P=0.802$).

The receiver-operating characteristic of each of the eight antigen/antibody systems in FS patients and the US controls

To define the relationships of specificity and sensitivity of each of the eight antigen/antibody systems using the index values obtained in the FS patients and the US controls, we generated receiver-operating characteristic (ROC) curves for each system (Figure 2). The ROC curves were generated by plotting sensitivity against 1−specificity, as the cutoff point is varied over the entire range of index values from a disease group (in this case, FS patients) and a negative control group (the US controls). The estimated area under the curve (AUC) is a summary of the entire ROC curve and is unaffected by choice of a cutoff point, as is the case for sensitivity and specificity. The AUC is interpreted as the probability that a randomly selected patient will have a higher value than a randomly selected healthy control for the test in question. The three highest estimated AUCs are for Dsg1 (0.98, 95% confidence interval (CI): 0.96–1.0), Dsc3 (0.90, 95% CI: 0.85–0.94), and Dsc1 (0.88, 95% CI: 0.84–0.93). It is noted that despite the high number of statistically significant differences found between FS patients and the US controls as depicted in Table 1, there is significant overlap in the distribution of index values in many of the antigen/antibody systems (Figure 1), which is reflected by low AUC values (Figure 2).

Percentage of serologically positive individuals in FS patients, endemic controls, and the US controls for each of the eight antigen/antibody systems tested

For each antigen/antibody system, we selected the cutoff point that would generate a specificity of 95%, i.e., false-positive rate of 5% among the US controls (Table 2). We defined all index values above the cutoff point as positive, and then determined the percentage of FS patients and endemic controls testing positive for each antigen/antibody system (Figure 3). Figure 3 shows the 5% false-positive rate among the US controls induced by the cutoff point selected. The FS patients show a high percentage (97%) of individuals with IgG reactivity to Dsg1. The FS patients also show a high percentage of individuals who test positive with each of the remaining seven antigens (∼40% for Dsg2, Dsg3, and Dsc1 and ∼50% for E-cadherin). Lower numbers of positive patients are found in the Dsg4 (∼30%), Dsc2 (∼20%), and Dsc3 (<20%) systems. A high percentage of endemic controls are also positive for the eight antigens tested: ∼55% for E-cadherin, ∼40% for Dsg1 and Dsc2, ∼30% for Dsc1, ∼28% for Dsg4, and ∼20% for Dsg2. Lower percentages of positive individuals are detected in the Dsg3 (∼14%) and Dsc3 (∼12%).

The correlation of index values of the eight antigen/antibody systems in FS patients and two healthy control groups

We next explored correlations among the responses to the eight antigens within each group or population to determine whether a higher index value to Dsg1 would be predictive of a higher index value to any of the other antigens within a particular group. High correlations among the responses

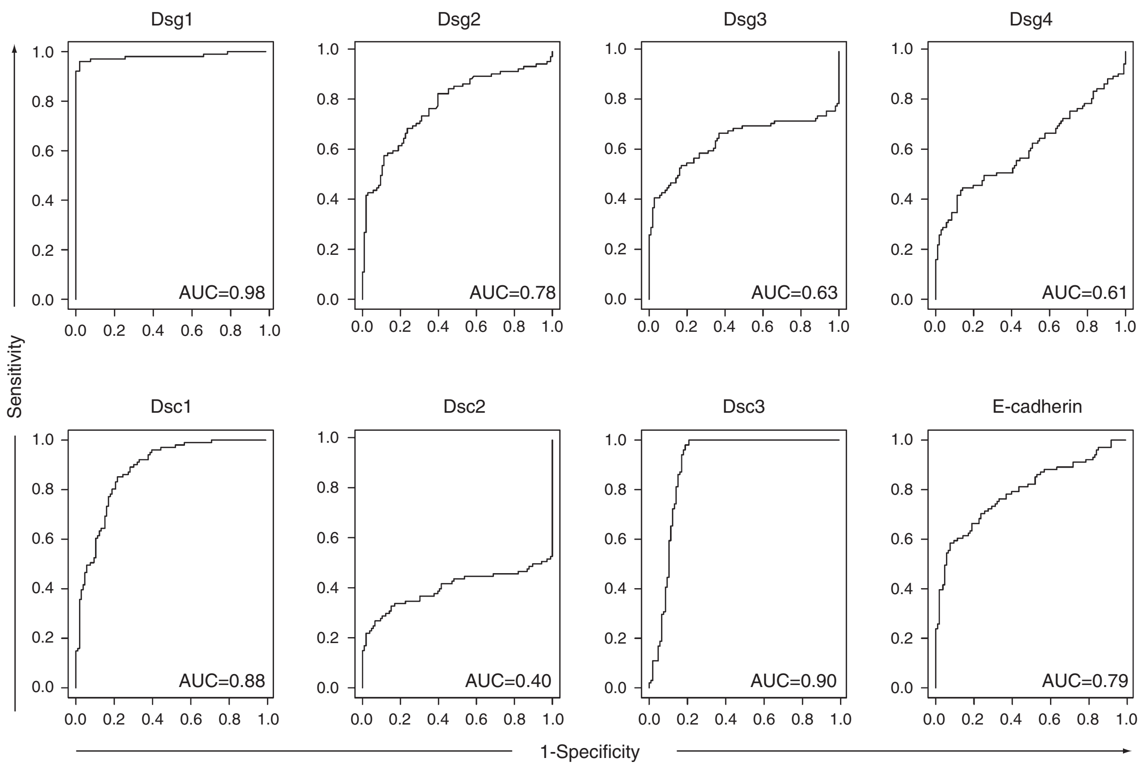

Figure 2. Receiver-operating characteristic (ROC) curves generated from healthy individuals from the United States (US controls) and fogo selvagem (FS) patient data. The ROC curve is a scatter plot of sensitivity against 1−specificity, as the cutoff point is varied over the entire range of index values. The area under the curves (AUCs) generated from these curves are shown in the right lower quadrant of each ROC box. The AUCs show that the highest performing antigen/antibody systems are Dsg1 (0.098), Dsc1 (0.88), and Dsc3 (0.90). Dsc, desmocollin; Dsg, desmoglein.

Table 2. Index value cutoff points selected for the eight antigen/antibody systems

Antigen	Cutoff point[1]	Sensitivity (%)	Specificity (%)
Dsg1	5	96	95
Dsg2	1.2	43	95
Dsg3	5	41	95
Dsg4	0.54	29	95
Dsc1	4	42	95
Dsc2	10	23	95
Dsc3	25	15	95
E-cadh	40	49	95

Abbreviations: Dsc, desmocollin; Dsg, desmoglein; E-cadh, E-cadherin.
[1]Cutoff points were generated by selecting a set specificity of 95%.

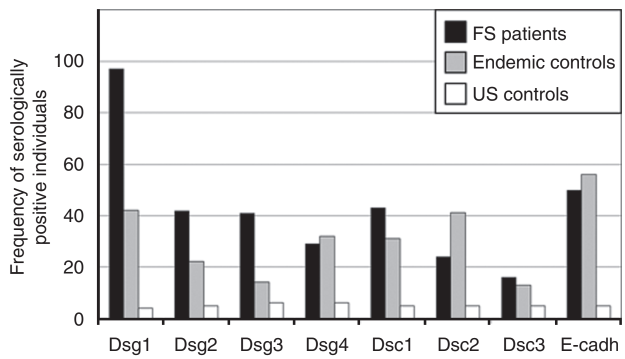

Figure 3. Percentage of serologically positive individuals among fogo selvagem (FS) patients, endemic controls, and healthy individuals from the United States (US controls) for each of the eight antigen/antibody systems. This bar graph represents positive sera as defined by the cutoff point. FS patients (black bars), endemic controls (gray bars), and the US controls (white bars) are shown. Dsc, desmocollin; Dsg, desmoglein; E-cadh, E-cadherin.

might suggest that the same pool of antibodies is responding to both antigens (cross-reactivity) or that a common stimulus is inducing both responses to the same magnitude. To determine whether there was correlation among the index values of the eight antigen/antibody systems within each population, scatter plots were created by plotting index values for one antigen against index values for each of the remaining antigens. Spearman correlations were then computed (r value shown in the right lower quadrant of each panel). As shown in Figure 4a, Dsg1 index values in FS patients are highly correlated with the Dsc1 ($r = 0.77$), Dsc3 ($r = 0.72$), and Dsg4 ($r = 0.70$) values. Thus, an FS patient with a high index value to Dsg1 tends to have a high index value to Dsc1, Dsc3, and Dsg4 as well. There are 28 pairwise correlations in each population of individuals. As a summary of these 28 correlations, the average correlation and range

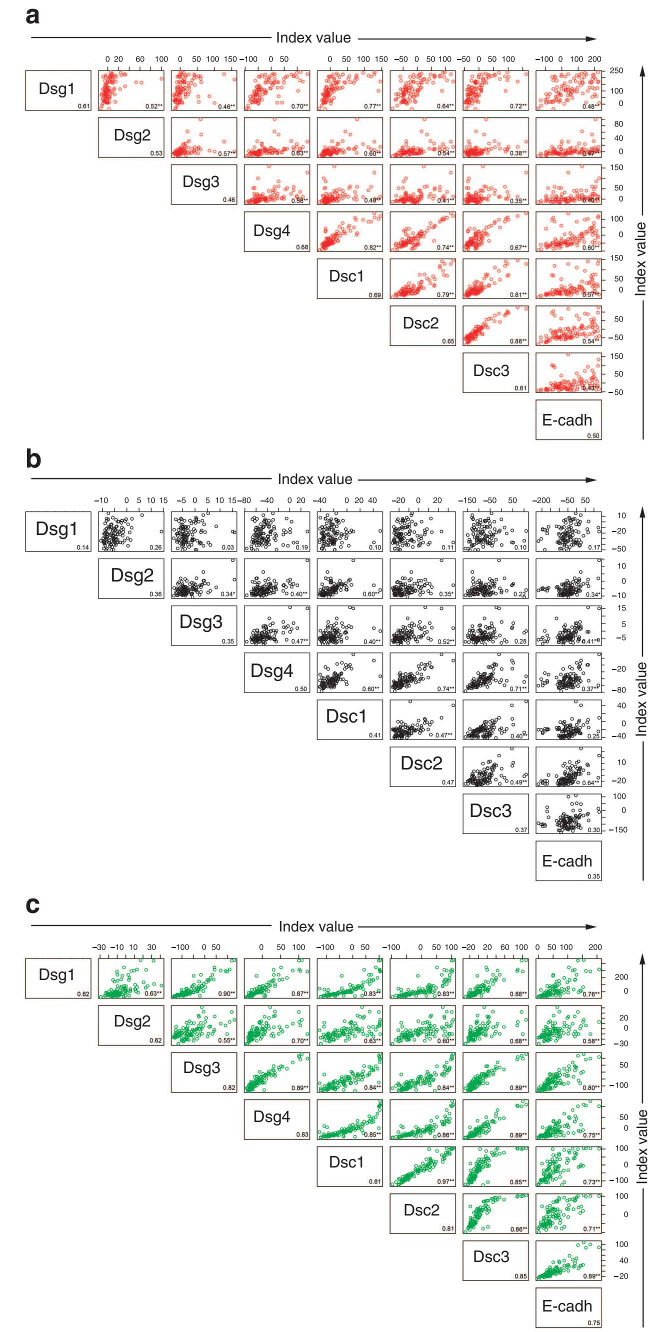

Figure 4. Correlation among the antibody responses. (**a**) Fogo selvagem (FS) patient, (**b**) healthy individuals from the United States (US control), and (**c**) endemic control antibody response correlations are depicted by index value scatter plots for each antigen/antibody system against the others. The Spearman correlation coefficient (*r* value) for each scatter plot is listed in the bottom right corner of each plot. Average *r* values for each antigen/antibody system against the remaining seven antigen/antibody systems are listed in the bottom right corner for each antigen. $^{*}P<0.05$, $^{**}P<0.01$, adjusting for multiple comparisons involving 84 *P*-values. Dsc, desmocollin; Dsg, desmoglein; E-cadh, E-cadherin.

were determined for each population of individuals. On average, the correlation among responses to the eight antigens in the FS patients is 0.59 (range 0.40–0.88; Figure 4a), but is lower in the US controls, with an average of 0.37 (range 0.03–0.74; Figure 4b). In contrast, the correlations among responses to the eight antigens in the endemic controls are strikingly high, with an average correlation of 0.79 (range 0.55–0.97; Figure 4c).

DISCUSSION

This investigation was undertaken to assess the spectrum of autoantibody responses to keratinocyte cadherins in the serum of FS patients and control individuals from the United States and from a highly endemic area of FS in Brazil. The three unique groups tested include the following: (1) FS patients who are already showing the pathogenic autoantibody response, perhaps secondary to sensitization to an environmental antigen(s); (2) normal immunologically naive individuals from a nonendemic area (i.e., the US control); and (3) individuals living in a Brazilian endemic region of FS, which may harbor the putative sensitizing antigen(s). This latter group may show autoantibody responses against keratinocyte cadherins that represent a transition between the previous two groups. A large number of FS and control sera were tested against the ectodomain of seven desmosomal cadherins and E-cadherin by ELISA. The results show a consistent and specific autoantibody response against these cadherins in FS patients and endemic controls. Moreover, the autoantibody responses to some of these cadherin antigens are correlated.

Three observations can be drawn from this investigation:

(a) The autoantibody response to the eight antigens in FS patients, the US controls, and endemic controls are unique and, in majority of the cases, significantly different in each of the three groups by 24 pairwise comparisons of the eight antigen/antibody systems. As expected, the index values of anti-Dsg1 autoantibodies from FS patients are significantly higher ($P<0.0001$) than that from the US controls, producing an AUC of 0.98 (95% CI: 0.98–1.00). These findings confirm that the ELISA assays for anti-Dsg1 autoantibodies as reported by our group (Warren *et al.*, 2000) and others (Amagai *et al.*, 1999) are reliable, sensitive, and specific, when used to distinguish FS patients and the US controls.

On comparing the index values of the FS patients and the endemic controls, we found that all index values are significantly different, with the exception of Dsc2 ($P=0.4033$) and E-cadherin ($P=0.2524$). The lack of a significant difference between the index values for Dsc2 and E-cadherin in FS patients and endemic controls reflects extensive overlap in the distributions of index values in the two groups.

Comparison of index values between the US controls and endemic controls reveals statistically significant differences for four antigen/antibody systems, Dsg3 ($P<0.0001$), Dsg4 ($P<0.0001$), Dsc3 ($P<0.0001$), and E-cadherin ($P<0.0001$). No significant differences are found for Dsg1 ($P=0.0162$), Dsg2 ($P=0.0150$), Dsc1 ($P=0.1119$), and Dsc2 ($P=0.820$). A reasonable explanation for these differences (or lack of differences) remains unknown; however, an unsubstantiated speculation would be that the earliest response in donors from endemic areas may be mediated by autoantibodies against the first group of antigens (Dsg3, Dsg4, Dsc3, and E-cadherin).

(b) As shown in Figure 2, ROCs of each antigen/antibody system provide a tool to evaluate the ability of each ELISA to separate known positive (FS patients) from known negative (the US controls) individuals across a range of cutoff points. An AUC of 0.50 indicates that the test is no better than chance at detecting a positive case, whereas values closer to 1.0 indicate an optimal test. The AUCs of Dsg1 (0.98, CI: 0.98–1.00), Dsc1 (0.88, CI: 0.84–0.930), and Dsc3 (0.90, CI: 0.85–0.95) showed that these ELISAs were the best separators of FS patients from the US controls. From our data, it is evident that aside from the Dsg1 ELISA, none of the remaining seven antigen/antibody ELISA systems would be useful as a diagnostic test for FS given their relatively low sensitivity.

ELISA cutoff points were chosen by setting a specificity of 95% to minimize false positives. Using these cutoffs (Figure 3), some FS patients tested positive for Dsg1 (97%), Dsg2 (42%), Dsg3 (41%), Dsc1 (43%), E-cadherin (50%), Dsg4 (29%), Dsc2 (24%), and Dsc3 (16%). The US controls show the 5% false-positive rate determined by the selected specificity. Interestingly, endemic controls show a sizable number of positive individuals in some of the antigen/antibody systems, i.e., E-cadherin (56%), Dsg1 (42%), Dsc2 (41%), Dsg4 (32%), and Dsc1 (31%). These findings suggest the relevance of the environment surrounding the FS patients and endemic controls as a source of sensitizing antigen(s) that lead to the production of these autoantibodies.

(c) Finally, the Spearman correlations between antigen/antibody systems of the three groups show interesting results. First, there is low correlation between anti-Dsg1 autoantibodies and any of the seven remaining antigen/antibody systems in the US controls. In contrast, anti-Dsg1 autoantibodies show strong correlation with Dsc1 and Dsc3 antibodies in FS patients. FS patients exhibit a weaker correlation between anti-Dsg1 autoantibodies and Dsg4, Dsc2, and E-cadherin. Endemic controls show striking correlations between anti-Dsg1 autoantibodies and autoantibodies to Dsg3, Dsg4, Dsc1, Dsc2, Dsc3, and E-cadherin.

The strong correlations seen in endemic controls and FS patients suggest exposure of these individuals to a common environmental antigen(s). The antigen(s) may not be available in environments where the US controls live. We acknowledge that it is difficult to distinguish between environmental and genetic influences on the autoantibody responses found in FS patients and endemic controls. Our current studies need further confirmation by establishing the autoantibody response of another control group composed of normal nonendemic Brazilian individuals of the same genetic stock as those of the Limao Verde endemic controls included in the current study. Should this new group generate results that do not overlap those of the endemic controls, then the environmental etiology of FS would be strengthened.

There may be several explanations for these findings:

(a) the putative environmental antigen(s) may share cross-reactive epitopes with all the keratinocyte cadherins, thus triggering independent populations of autoantibodies with specificities to each of the eight antigens;
(b) one or all of the environmentally elicited antikeratinocyte cadherin autoantibodies recognize cross-reactive epitopes on the eight antigens tested because of structural homologies among these keratinocyte cadherins; and
(c) finally, it is feasible that once an initial autoantibody response is triggered against one of these cadherins (primary response), this response may be followed by autoantibody responses against other keratinocyte cadherins by the epitope-spreading mechanism. However, as these results represent a single time-point observation, determination of the progression of these responses over time (as seen in epitope spreading) is not possible with this data set.

As FS is a rare occurrence, it is likely that other factors such as the HLA may facilitate the development of this disease. It is intriguing that anti-E-cadherin autoantibodies are detected in a large number of individuals from the healthy endemic control (58%) and FS group (42%), raising the hypothesis that this primitive molecule may be the initial cross-reactive antigen. Much work is needed to test these appealing speculations.

MATERIALS AND METHODS

Sources of sera

A total of 313 sera from adult donors were tested for IgG autoantibodies against seven desmosomal cadherins and E-cadherin (101 sera were obtained from FS patients, 106 from healthy controls from the United States, and 106 from healthy controls living in the Limao Verde endemic area of Brazil). FS sera were collected from patients originating from endemic regions, but hospitalized at the time of drawing blood in three Brazilian hospitals dedicated to treat these patients: Hospital das Clinicas, Sao Paulo ($n=25$); Hospital de Doenças Tropicaes, Goiania ($n=31$); and Hospital Adventista do Penfigo, Campo Grande ($n=45$). The disease was confirmed by clinical, histological, and immunofluorecent findings. The patients were in different clinical stages of evolution and were undergoing systemic steroid therapy. The indirect immunofluorescence studies showed anti-epidermal intercellular substance autoantibodies in titers above 1:80 in 94 patients, 1:40 in 5 patients, 1:20 in 1 patient, and negative in 1 patient. Healthy control sera (defined as complete absence of cutaneous disease) were obtained from blood bank donors from the University of North Carolina Blood Bank and healthy individuals from Limao Verde, Brazil.

The study was approved by the University of North Carolina Institutional Review Board, and conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

Construction and production of recombinant Dsg1, Dsg3, and E-cadherin antigens

We have previously constructed and expressed the entire extracellular domains of human Dsg1, Dsg3, and E-Cadherin using the baculovirus system (Ding *et al.*, 1997, 1999; Diaz *et al.*, 2008). Soluble ectodomains of Dsg1, Dsg3, and E-cadherin were produced in High Five (Invitrogen, Carlsbad, CA) insect cells by infection with high-titer recombinant baculovirus stocks. Optimal infection conditions for each recombinant protein were determined by time-course studies (Liebman *et al.*, 1999). The average protein yield was $10\,\mu g\,ml^{-1}$ of culture supernatant.

Subcloning of the Dsg2 ectodomain into a baculovirus expression vector

Complementary DNA encoding the entire human Dsg2 was provided by Dr M. Mahoney, Jefferson Medical School, and was used to construct the human Dsg2 ectodomain. The DNA of the Dsg2 ectodomain was amplified by PCR using the primer pair of 5′-ATCGGG CGCGGATCCATGGCGCGGAGCCCGGG-3′ and 5′-ATTCGAAAG CGGCCGCCTAGTGATGGTGATGATGGTGCAGGCCCACATAGG AGTCAT-3′, and was inserted into pFastBacI (Gibco Life Technologies, Gaithersburg, MD) at *Bam*HI and *Not*I sites. The coding region of the construct begins with the endogenous initial Met followed by a putative signal sequence, as well as an in-frame His-tag at the C terminus to ensure the secretion, and to facilitate the purification and detection of the expressed antigen. The resulting plasmid, pFastBacI-Dsg2 ectodomain, was used to construct into the Bac-to-Bac system following the manufacturer's protocol. Virus stock was prepared by infecting sf9 insect cell in Grace's insect cell medium (Invitrogen) containing 10% fetal bovine serum at 27 °C. Viral titers were determined by end-point dilution assay in sf9.

Expression of Dsc1, Dsc2, and Dsc3 and Dsg4 recombinant antigens

The entire complementary DNA of the ectodomain of Dsc1, Dsc2, and Dsc3 constructed in the pEVmod was provided by Dr Takashi Hashimoto, Department of Dermatology, Kurume University, Kurume, Japan. These complementary DNAs were subcloned into the BD BaculoGold virus vector (BD Biosciences, San Diego, CA).

The Dsg4 extracellular domain already subcloned into the baculovirus vector was kindly provided by Dr Masayuki Amagai, Department of Dermatology, Keio University School of Medicine, Tokyo, Japan.

Production and purification of recombinant E-cadherin and desmosomal cadherins

His-tagged recombinant forms of Dsg1–4, Dsc1–3, and E-cadherin ectodomains were generated in the baculovirus expression system and purified by nickel affinity chromatography using the procedure previously described (Ding *et al.*, 1997).

ELISA

Immunomicrotiter plates (Costar, Cambridge, MA) were coated with one of the eight purified desmosomal cadherins (200 ng per well for Dsc1, Dsg1, Dsg3, and E-cadherin; 100 ng per well for Dsg2 and Dsc2; and 50 ng per well for Dsg4 and Dsc3) at 4 °C overnight. After washing five times with Tris-buffered saline containing 5 mM Ca^{++} and 0.05% Tween-20 (TBS/Ca^{++}/T-20), the plate was blocked with 1% BSA in TBS/Ca^{++}/T-20 at room temperature for 1 hour. The plate was then washed five times and incubated with duplicate samples of diluted serum for 1 hour at room temperature. Following washes, the plate was incubated with diluted horseradish peroxidase–conjugated goat anti-human IgG (Bio-Rad, Hercules, CA) for 1 hour (1:1,000 for seven desmosomal antigens and 1:1,500 for E-cadherin). The color development was achieved with the peroxidase substrate o-phenylenediamine.

An FS serum (1:100 dilution) that produced consistent and reproducible positive optical density (OD) values for IgG anti-Dsg1 autoantibodies was selected as a positive control. We also selected a positive serum (1:100 dilution) for Dsg2 (CG14), Dsg3 (Dip), Dsg4 (CG42a), Dsc1, Dsc2, and Dsc3 (CG42a) and E-cadherin (1:200 dilution). A normal human serum from the United States was used as a negative control throughout. Results were expressed as index value units as reported by Amagai *et al.* (1999) and Diaz *et al.* (2008). The index value was defined in terms of OD as follows:

$$\text{Index value} = \frac{(\text{Test sample OD}) - (\text{Negative control OD})}{(\text{Positive control OD}) - (\text{Negative control OD})} \times 100$$

Negative index values occur when the test sample OD is less than the negative control OD.

As a reference, we provide the average ODs of duplicate tests of negative and positive control sera used to generate index values: Dsg1 (negative 0.81, positive 1.60), Dsg2 (negative 0.48, positive 1.92), Dsg3 (negative 1.39, positive 2.55), Dsg4 (negative 0.78, positive 2.45), Dsc1 (negative 1.76, positive 3.16), Dsc2 (negative 1.65, positive 3.25), Dsc3 (negative 0.68, positive 2.70), and E-cadherin (negative 0.17, positive 1.36).

Statistical analysis

Nonparametric methods (DeLong *et al.*, 1988) were used for the estimation and testing of ROC curves (Hanley and McNeil, 1982) and AUCs. Spearman correlation was used to assess the strength of correlation among the various measures. The R software (R Development Core Team, 2011), version 2.13.0, was used to generate the graphs. The data analysis was performed using the SAS software. Copyright, SAS Institute. SAS and all other SAS Institute product or service names are registered trademarks or trademarks of SAS Institute, Cary, NC.

The Wilcoxon rank sum test (Hollander and Wolfe, 1999) is a statistical hypothesis test of equality of two distributions (the distributions in the two groups). Briefly, the test is performed by pooling the two samples into one sample of n observations, ranking the values from smallest (rank = 1) to largest (rank = n), computing the mean rank in each group, and then comparing the mean ranks in a formal statistical manner. It is somewhat similar in spirit to the two-sample *t*-test, but it only takes the ordering of the observations into account, not their actual magnitude. Assumption of normal distributions is not required.

Adjustment for multiple comparisons controls the overall type-I error when a large number of hypothesis tests are performed. For example, when 20 hypothesis tests are performed at the 0.05 level each, and all 20 null hypotheses are in fact true, on average, one test turns out statistically significant ($P < 0.05$), and the probability of one or more significant tests is larger than 0.05, and can be much larger. In fact, if the 20 tests are independent, there is a 64% chance of at least one test showing a significant result when all 20 null hypotheses are true. Controlling the overall type-I error guarantees that the probability of one or more significant findings ($P < 0.05$), when all 20 null hypotheses are in fact true, is 0.05 or less. The Bonferroni method achieves that control by performing each test at the 0.05/20 = 0.0025 level, i.e, the overall type-I error is divided by the number of tests. The method does not require the tests to be independent (Farcomeni, 2008).

CONFLICT OF INTEREST
The authors state no conflict of interest.

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