The IgM Anti-Desmoglein 1 Response Distinguishes Brazilian Pemphigus Foliaceus (Fogo Selvagem) from Other Forms of Pemphigus

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Fogo selvagem (FS) and pemphigus foliaceus (PF) possess pathogenic IgG anti-desmoglein 1-(Dsg1) autoantibodies. Although PF occurs sporadically, FS is endemic in Limao Verde (LV), Brazil (3.4% prevalence). IgM anti-Dsg1 were detected in 58% FS LV patients ($n=31$), 19% of FS patients from Hospital-Campo Grande ($n=57$), 19% from Hospital-Goiania ($n=42$), 12% from Hospital-Sao Paulo ($n=56$), 10% of PF patients from United States ($n=20$), and 0% of PF patients from Japan ($n=20$). Pemphigus vulgaris ($n=40$, USA and Japan), bullous pemphigoid ($n=40$, USA), and healthy donors ($n=55$, USA) showed negligible percentages of positive sera. High percentages of positive IgM anti-Dsg1 were found in healthy donors from four rural Amerindian populations (42% of 243) as compared with urban donors (14% of 81; $P<0.001$). More than 50% of healthy donors from LV ($n=99$, age 5–20 years) possess IgM anti-Dsg1 across ages, whereas IgG-anti-Dsg1 was detected in 2.9% (age 5–10 years), 7.3% (age 11–15 years), and 29% of donors above age 16. IgM anti-Dsg1 epitopes are Ca^{2+} and carbohydrate-independent. We propose that IgM anti-Dsg1 are common in FS patients in their native environment and uncommon in other pemphigus phenotypes and in FS patients who migrate to urban hospitals. Recurrent environmental antigenic exposure may lead to IgM and IgG responses that trigger FS.

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Journal of Investigative Dermatology (2008) **128,** 667–675; doi:10.1038/sj.jid.5701121; published online 25 October 2007

INTRODUCTION

Fogo selvagem (FS) and pemphigus foliaceus (PF) share clinical and histological features, and are characterized serologically by pathogenic IgG4 autoantibodies against desmoglein 1 (Dsg1) (Rock *et al.*, 1989; Stanley *et al.*, 1986; Diaz *et al.*, 1989a). PF occurs sporadically in North America, Europe, and Asia, whereas FS is endemic among poor laborers of all races and both sexes living in certain endemic regions of Brazil (Diaz *et al.*, 1989b).

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Abbreviations: BP, bullous pemphigoid; Dsg1, desmoglein 1; FS, fogo selvagem; LV, Limao Verde; mcPV, mucocutaneous pemphigus vulgaris; mPV, mucosal pemphigus vulgaris; OD, optical density; PF, pemphigus foliaceus; PV, pemphigus vulgaris

Received 12 April 2007; revised 15 August 2007; accepted 23 August 2007; published online 25 October 2007

The Amerindian reservation of Limao Verde (LV), located in the state of Mato Grosso do Sul, Brazil, is the home of ~1,200 members of the Terena tribe of Amerindians, and is an active focus of FS, exhibiting a 3.4% prevalence of disease (Figure 1) (Hans-Filho *et al.*, 1996). For the past 12 years, we have periodically collected clinical and serological data from FS patients as well as clinically normal individuals residing in and around this settlement. We have found that the autoantibody response against Dsg1 in healthy individuals from LV and neighboring communities is common, and directly related to proximity to this reservation (Warren *et al.*, 2000; Hilario-Vargas *et al.*, 2006). Over the course of this investigation, we have also observed the clinical and serological conversion from normal-to-disease state in 10 individuals (Warren *et al.*, 2000; Li *et al.*, 2003). These conversions were marked by a transition from IgG1 autoantibodies targeting the EC5 domain of Dsg1 during the preclinical stage to a predominantly IgG4-mediated autoantibody response against the Dsg1 EC1-2 domains with the onset of clinically active disease (Li *et al.*, 2003; Warren *et al.*, 2003).

Several studies support the concept that the autoimmune response in FS is triggered by an exposure to, an as yet unknown, environmental antigen(s). Previous epidemiological studies have shown that the incidence of FS tends to

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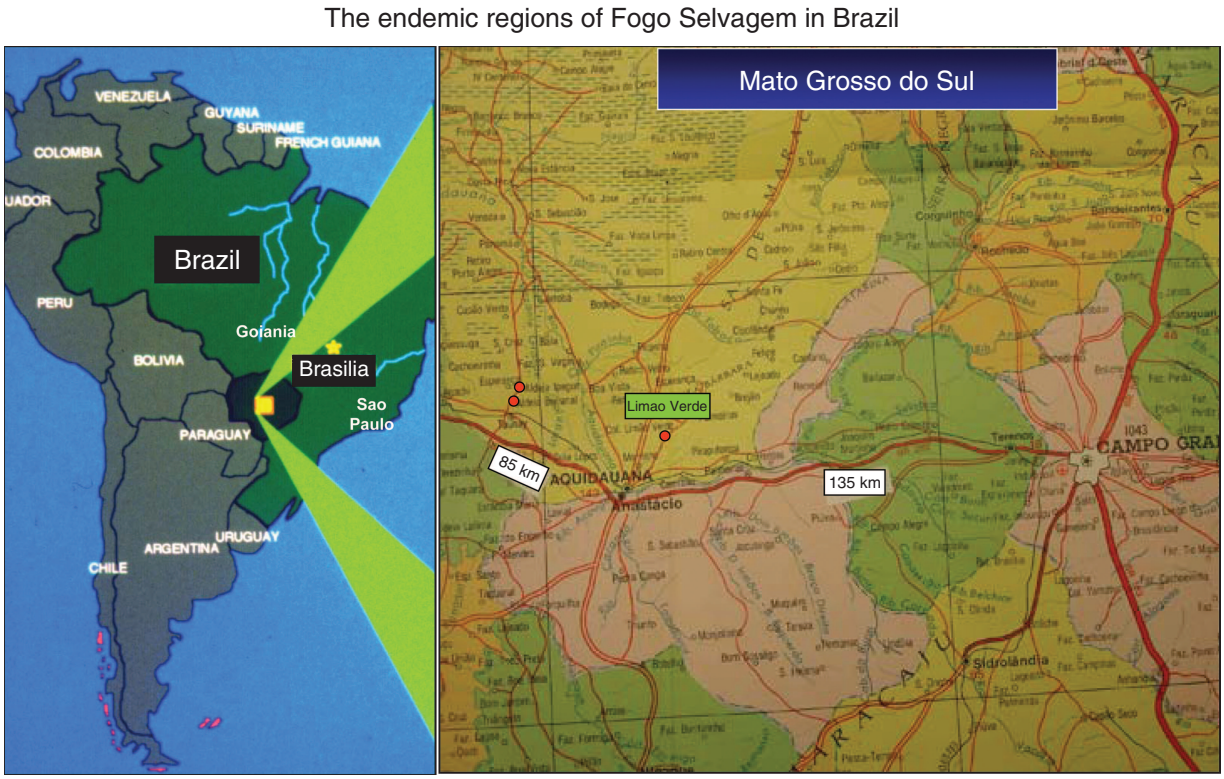


Figure 1. Map of South America and Brazil depicting sites of donor serum collection. These sites are located in known endemic regions of FS. Specialized hospitals in the cities of Sao Paulo (state of Sao Paulo), Goiania (state of Goias), Brasilia (capital of Brazil), and Campo Grande (state of Mato Grosso do Sul) are referral centers for FS patients from the central planes of Brazil. LV, with a population of ~1,200 individuals is an active focus of FS (3.4% prevalence) and is located in the state of Mato Grosso do Sul. The Amerindian reservations of Bananal, Ipegue, and Agua Branca are located 85 km west of LV and are inhabited by the same Terena tribes who live in LV. The Bananal and Ipegue reservations have an estimated population of 5,000 individuals. Aquidauana city with a population of 40,000 inhabitants is located 25 km southwest of LV. Campo Grande (state capital) has a population of 300,000 inhabitants and is located 135 km east of Aquidauana. The city of Sao Paulo with a population of 12 million is located approximately 1000 km east of Campo Grande. Goiania city has a population of 400,000 and is located 900 km northeast of Campo Grande.

decrease as living conditions of endemic populations improve, as observed in the states of Sao Paulo and Parana over the past four decades (Diaz *et al.*, 1989b; Empinotti *et al.*, 2006). Additional seroepidemiological studies suggest that exposure to blood feeding arthropods such as simuliids, bed bugs, and reduvid bugs, as well as poor housing are potential risk factors of FS (Aoki *et al.*, 2004). Furthermore, the sera of patients with leishmaniasis, onchocerciasis, and Chagas disease contain significant titers of anti-Dsg1 autoantibodies specific for the EC5 domain, suggesting that arthropod antigens may cross-react with epidermal Dsg1, thus triggering autoantibody formation in individuals exposed to these arthropods (Diaz *et al.*, 2004). It should be stressed that attempts to isolate or directly associate FS to bacterial and viral infections have been unsuccessful to date (Diaz *et al.*, 1989a, b).

In an effort to detect unique serological markers that may distinguish FS from PF, sera from patients with FS and PF as well as clinically normal individuals from Brazil, United States, and Japan were tested for the presence of IgM anti-Dsg1 autoantibodies using ELISA. These studies were facilitated by the availability of serum samples from Brazilian hospitals and highly endemic regions of FS in Brazil (depicted in Figure 1). Findings suggest that the IgM anti-Dsg1 autoantibody response is a distinct marker of antigenic exposure within an endemic area, and is prevalent in FS patients as well as clinically normal individuals living in LV and its neighboring rural regions. It is hypothesized that the IgM anti-Dsg1 autoantibody response in humans exposed to an environmental antigen(s) may represent the earliest event in the sensitization process that leads to FS.

RESULTS

Anti-Dsg1 IgM and IgG autoantibodies in FS and non-endemic PF

We first evaluated the IgM and IgG anti-Dsg1 response in FS and PF patients segregated in groups. The first group included sera from FS patients living in their native environment, LV. Other groups included sera from FS patients admitted in three Brazilian hospitals located in the cities of Campo Grande (CG, state of Mato Grosso do Sul), Goiania (GO, state of Goias), and Sao Paulo (SP, state of Sao Paulo). The remaining two groups comprise PF patients from the US (PF-USA) and Japan (PF-Japan). The percentages of individuals showing positive IgM (cutoff above 50) and IgG (cutoff above 10) anti-Dsg1 index values in each of these groups are shown in Figure 2, while Figure S1 provides additional information about the distribution of index values in these groups. The specificity of the IgM reactivity of the FS/PF sera with Dsg1

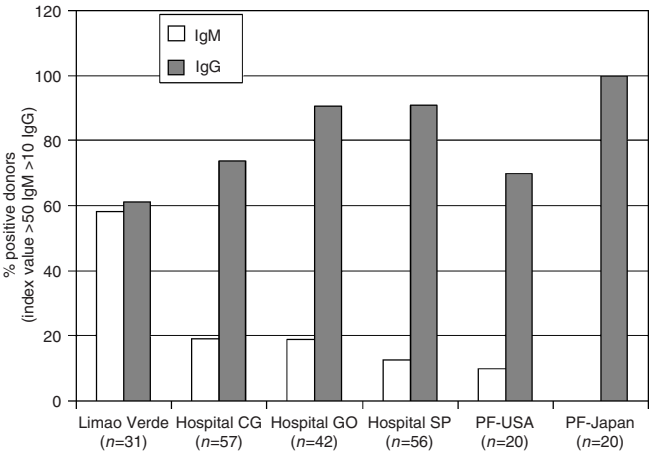


Figure 2. IgM and IgG anti-Dsg1 reactivity of sera from patients with FS and PF. White columns show the percentages of donors from each group (LV, CG, GO, SP, PF-USA and PF-Japan) possessing positive values of IgM anti-Dsg1 autoantibodies and dark columns show the percentages of donors possessing positive values of IgG anti-Dsg1 autoantibodies. The number of donors in each group is included under each site.

was also investigated by testing positive IgM anti-Dsg1 sera ($n=8$) with an unrelated epidermal antigen recognized by bullous pemphigoid (BP) sera by ELISA, that is the BP180 antigen (Liu and Diaz, 2001). We found no significant IgM reactivity against this antigen in these sera. This finding is in agreement with early reports from our group where we found no IgG reactivity of FS sera with either BP180 antigen (Warren *et al.*, 2000) or antinuclear antibodies (Squiquera *et al.*, 1988). Additionally, the Dsg1 ELISA for IgA anti-Dsg1 autoantibodies in 15 sera tested, including the normal human USA sera ($n=7$), PF serum ($n=1$), FS sera ($n=5$), and healthy Amerindian donors from Brazil ($n=2$), showed negative results. Finally, the results obtained by two independent investigators testing for IgM anti-Dsg1 autoantibodies in a set of 48 positive and negative coded sera demonstrated a perfect linear relationship (correlation coefficient 0.977, $P<0.001$).

As shown in Figure 2 (white columns) and Figure S1A in LV, a highly endemic focus of FS, 58% of 31 cases were positive for IgM anti-Dsg1 autoantibodies. We found no correlation of IgM anti-Dsg1 autoantibodies with age or sex of 31 FS patients. Of 10 FS patients ranging in age from 15 to 25 (four females and six males), sera from six demonstrated IgM anti-Dsg1 autoantibodies. Sera from 10 of 17 patients age 26–50 years (10 females and 7 males) had IgM anti-Dsg1 autoantibodies. Finally, positive IgM serology was detected in 2 of 4 FS patients age 60–80 years. Of the 31 FS patients, 8 were in clinical remission and 3 of these patients showed positive IgM anti-Dsg1 autoantibodies. Fifteen of 23 FS patients (65%) with active clinical disease showed positive IgM anti-Dsg1 serology. In contrast, the percentage of positive IgM anti-Dsg1 sera was lower in patients staying in urban hospitals away from their native environment. Only 19% of 57 patients admitted to Hospital-CG, 19% of 42 cases from Hospital-GO, and 12% of 56 patients from Hospital-SP were positive. Similarly, in only 10% of 20 PF-USA patients and none of 20 PF-Japan donors were positive. Significant heterogeneity exists in the prevalence of IgM antibodies between the six different settings ($P>0.001$). However, most of the differences were between LV, the hospital-based group (CG, GO, and SP), and the PF group (USA and Japan). The differences among the three hospital-based groups were small ($P=0.56$), as was the difference between PF-USA and PF-Japan ($P=0.15$).

The serum samples from the six groups were also tested by ELISA for IgG anti-Dsg1 autoantibodies. As shown in Figure 2, dark columns, and Figure S1B, IgG autoantibodies were detected in 61% of FS patients from LV, 75% from Hospital-CG, 90% from Hospital-GO, 91% from Hospital-SP, 70% of 20 PF-USA, and 100% of 20 PF cases from Japan. These results are consistent with what is known in the literature, that is IgG anti-Dsg1 autoantibodies are diagnostic markers of FS and rough indicators of disease activity (Rock *et al.*, 1983; Stanley *et al.*, 1986; Diaz *et al.*, 1989a).

To test if binding of IgG anti-Dsg1 autoantibodies would interfere with the binding of IgM anti-Dsg1 autoantibodies, five representative high-titer FS sera were tested by ELISA double dilutions (1:100, 1:200, 1:400, and 1:800), and estimated IgM and IgG index values were obtained. No changes in the index values of IgM and IgG autoantibodies were observed across all dilutions. Additionally, microtiter wells coated with recombinant Dgs1 were preincubated with a high-titer IgG anti-Dsg1 serum before testing a set of IgM anti-Dsg1-positive sera ($n=4$). No changes in IgM index values in these sera were observed.

Anti-Dsg1 IgM and IgG autoantibodies in pemphigus vulgaris and BP

To investigate further the disease specificity of the anti-Dsg1 response, we tested the sera of patients with other cutaneous autoimmune diseases. One group comprises patients with pemphigus vulgaris (PV), a potentially lethal organ-specific autoimmune disease characterized by mucosal and/or skin erosions and blisters and autoantibodies against Dsg3 (Amagai *et al.*, 1991). One clinical variant of PV is characterized by mucosal lesions (mucosal pemphigus vulgaris (mPV)) and the other exhibits mucocutaneous lesions (mucocutaneous pemphigus vulgaris (mcPV)) (Ding *et al.*, 1997; Amagai *et al.*, 1999). Whereas IgM anti-Dsg1 autoantibodies have never been reported in the sera of PV patients, IgG anti-Dsg1 autoantibodies are commonly detected in mcPV (Ding *et al.*, 1999). In this study we found IgM anti-Dsg1 autoantibodies in 0% of 10 mPV and 0% of 10 mcPV patients from United States and in 5% of 20 mPV and 5% of 20 mcPV from Japanese patients. IgG anti-Dsg1 autoantibodies were detected in 50% of 10 mcPV sera from United States and in 75% of 20 mcPV patients from Japan. These IgG anti-Dsg1 autoantibodies were absent in the sera of mPV from United States ($n=10$) and Japanese ($n=20$) patients. We also studied the sera of another organ-specific autoimmune skin disease, BP, which is characterized by subepidermal blisters and autoantibodies against two hemidesmosomal antigens, that is BP230 and BP180 (Liu and Diaz, 2001). We found IgM anti-Dsg1 autoantibodies in 5%

of 40 BP patients from United States. IgG anti-Dsg1 autoantibodies were detected in 10% of these cases. These results further demonstrate the restricted IgM anti-Dsg1 response in FS patients living in LV.

Anti-Dsg1 IgM and IgG autoantibodies in clinically healthy individuals from rural and urban populations

To further investigate the prevalence of IgM and IgG anti-Dsg1 autoantibodies in populations prone to develop FS, we tested the sera of healthy Amerindians living in rural endemic areas of FS in Brazil. These results were compared with the sera from normal individuals living in a semi rural city (Aquidauana) and other Brazilian cities distant from LV, the endemic focus of FS. As shown in Figure 3a (white columns) the sera of donors from rural areas (LV, Bananal, Ipegue, and Agua Branca) exhibit higher prevalence of IgM anti-Dsg1 autoantibodies than those donors from urban communities (Aquidauana, Campo Grande, and Sao Paulo) and United States. Figure S2A provides additional information about the distribution of index values. The heterogeneity in the proportions of positive IgM anti-Dsg1 sera among the four urban donors ($P=0.04$) was more marked than among the rural individuals ($P=0.09$). The prevalence of IgM anti-Dsg1 autoantibodies were 42% in the rural group and 14% in the urban group ($P<0.001$).

The prevalence of positive IgG anti-Dsg1 sera was also higher among healthy Amerindians from rural areas of LV, Bananal, Ipegue, and Agua Branca as compared with urban donors of Aquidauana, Campo Grande, Sao Paulo, and United States (Figure 3a, dark columns, and Figure S2B). These results confirm that IgM and IgG anti-Dsg1 autoantibodies are also present in the sera of healthy individuals living in rural areas of Brazil, where FS is endemic (Warren *et al.*, 2000; Hilario-Vargas *et al.*, 2006). Additionally, we have shown that these rural populations, settled in endemic areas of FS, possess IgG anti-Dsg3 autoantibodies (markers of PV) and in a few of these individuals an ''endemic'' form of mcPV may develop (Rocha-Alvarez *et al.*, 2007).

The IgM and IgG anti-Dsg1 response from neonatal, childhood to early adulthood

We also tested the IgM and IgG anti-Dsg1 response from childhood to adulthood in a large group of normal donors age 5–20 years ($n=99$) from LV. As shown in Figure 3b, 53% of individuals of the first cohort (age 5–10 years, $n=34$), 51% of the second (age 11–15 years, $n=41$), and 62.5% of the third cohort (age 16–20 years, $n=25$) exhibited positive IgM anti-Dsg1 results. Of interest, the IgG anti-Dsg1 response was detected in 2.9% of donors of the first cohort, 7.3% of the second cohort, and 29% of the third cohort. Figure S3 shows index values of IgM and IgG anti-Dsg1 autoantibodies plotted against age. The sample size in these analyses is 98. This analysis excluded one IgG index value above 80 from a 19-year-old subject who was later diagnosed with FS. Linear regression models were used in the analysis of trends in IgM and IgG levels with age. There was no significant trend in the IgM index values ($P=0.76$), but the trend for IgG index values was significant. The mean IgG index is estimated to increase by 0.45 units with each year of age, 95% confidence interval (0.032, 0.87), $P=0.037$.

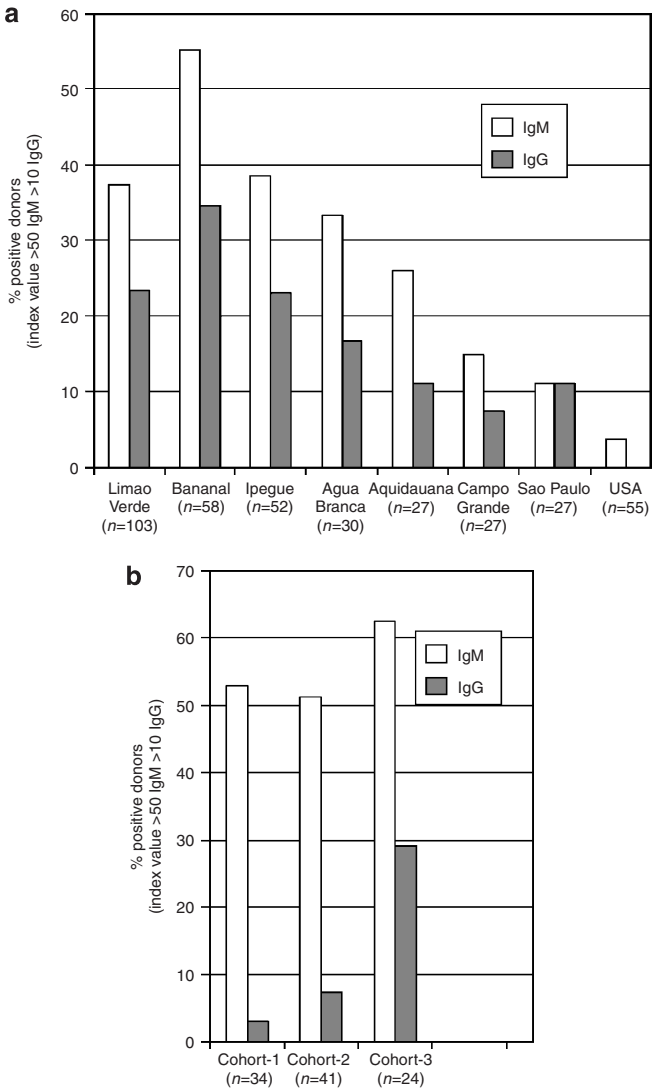


Figure 3. IgM and IgG anti-Dsg1 reactivity of sera from healthy control individuals. (**a**) Prevalence of IgM and IgG anti-Dsg1 autoantibodies in sera from donors of rural and urban sites. White columns show the percentages of donors of each group possessing positive values of IgM anti-Dsg1 autoantibodies. Rural donors: Limao Verde, Bananal, Ipegue, and Agua Branca. Urban donors: Aquidauana, Campo Grande, Sao Paulo, and United States. The dark columns show the percentages of donors possessing IgG anti-Dsg1 autoantibodies (same subjects). The number of donors in each site is provided at the bottom of each site. (**b**) IgM and IgG anti-Dsg1 reactivity of sera from healthy donors age 5–20 years old sorted out in three age groups (age 5–10, 11–15, and 16–20 years old). The percentages of donors possessing positive IgM anti-Dsg1 values (white columns) and the percentage of donors of each cohort possessing IgG anti-Dsg1 autoantibodies (dark columns). One donor who showed the highest index value for IgG anti-Dsg1 in cohort-3 developed FS during the course of this study. The number of donors is provided at the bottom of each cohort.

Sera from eight pairs of mothers with FS and their neonates were tested for IgG and IgM anti-Dsg1 autoantibodies by ELISA (Rocha-Alvarez *et al.*, 1992). We found that mothers'

sera showed high index values of IgG and IgM anti-Dsg1 autoantibodies. The neonates, however, showed negative index values of IgM autoantibodies (data not shown).

IgM anti-Dsg1 autoantibodies bind calcium and N-glycosylation-independent epitopes

To test the effects of calcium and N-glycosylation on IgM binding to Dsg1, we selected a set of FS sera and a set of healthy individuals, known to have IgM anti-Dsg1 autoantibodies, to investigate whether these antibodies recognize Ca^{2+}-dependent epitopes or carbohydrate epitopes on Dsg1. Deglycosylated Dsg1 was prepared in the baculovirus system by incorporating tunicamycin in the expression step (Amagai *et al.*, 1995). The deglycosylated Dsg1 was further purified by concanavalin-A agarose affinity chromatography by collecting the unbound fraction. Deglycosylated Dsg1 showed the respective reduction on molecular weight by SDS–PAGE (data not shown). The glycosylated Dsg1 was tested by ELISA in the presence of Ca^{2+} (routinely added to Tris-buffered saline used in ELISA procedures) or in the absence of Ca^{2+} (5 mM EDTA added to Tris-buffered saline). The results show that IgM anti-Dsg1 autoantibodies continue to bind Dsg1 in the absence of Ca^{2+} (Figure 4a); in fact the values were higher ($P=0.0042$). Similarly, IgM anti-Dsg1 autoantibodies from FS patients and healthy individuals continue to bind deglycolsylated Dsg1 (Figure 4b). On the contrary, EDTA completely abrogates the IgG anti-Dsg1 autoantibodies reactivity with Dsg1 (data not shown). These results demonstrate that IgM anti-Dsg1 and the IgG anti-Dsg1 autoantibody systems present in the sera of FS patients and normal individuals are distinct.

IgM anti-Dsg1-positive sera do not stain the epidermis by indirect immunofluorescence, but immunoprecipitate Dsg1

Twenty sera from the following individuals were selected: (a) FS ($n=3$) and PF ($n=1$), and (b) healthy donors ($n=16$) exhibiting high index values of IgM anti-Dsg1 autoantibodies by ELISA. Ten normal donors from Amerindian reservations had low index values for IgG anti-Dsg1, and six had high index values of IgG anti-Dsg1. The four sera from patients comprising FS and PF had high index values of IgG anti-Dsg1 by ELISA. All 20 serum samples produced negative indirect immunofluorescence (IF) staining of the intercellular spaces of human skin and monkey esophagus for IgM autoantibodies. The sera of the four FS and PF patients stained the human skin and monkey esophagus epithelial cell surfaces, producing the classic pemphigus pattern using FITC-labeled anti-human IgG conjugate.

The sera from four patients (three FS and one PF) who possessed IgM anti-Dsg1 autoantibodies by ELISA also recognized Dsg1 by immunoprecipitation/immunoblot analysis as shown in Figure 5 (lanes 4, 7, 9, and 10). The sera of three normal donors from Amerindian reservations (lanes 5, 6, and 8) exhibiting IgM autoantibodies by ELISA also bind Dsg1 by IP. The same Dsg1 peptide was bound by IgG autoantibodies from a patient with FS (lane 2).

DISCUSSION

Immunization of naive individuals to a particular antigen usually triggers a primary immune response characterized by low-affinity IgM antibodies. Re-exposure to the same antigen subsequently precipitates a sustained secondary response characterized by high-affinity IgG antibodies (Abbas and Lichtman, 2005). In humans, detection of antigen-specific IgM antibodies in sera may be an indicator of a recent infection or re-infection with viral diseases such as rubella (Hamkar *et al.*, 2005), infectious mononucleosis (Obel *et al.*, 1996), or hepatitis B (Chu, 2006); bacterial infections such as Lyme disease (Cermakova *et al.*, 2005); or parasitosis such as toxoplasmosis (Montoya, 2002; Reis *et al.*, 2006). In FS, however, the etiology is obscure and there is no experimental or clinical evidence that the disease may be linked to an infectious or parasitic disease (Diaz *et al.*, 1989a, b; Aoki *et al.*, 2004). It is also known that certain number of healthy,

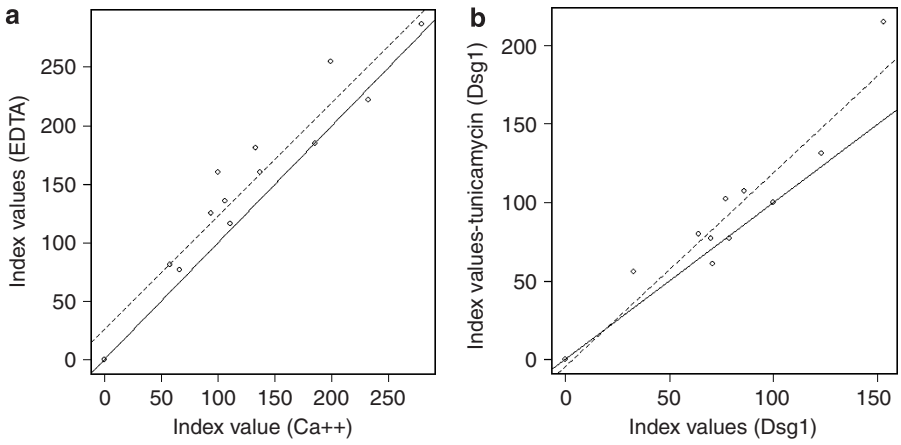


Figure 4. Effect of Ca^{2+} and Dsg1 glycosylation on the binding of IgM anti-Dsg1 autoantibodies. Microtiter wells were coated with 200 ng of (**a**) recombinant glycosylated Dsg1 and (**b**) deglycosylated Dsg1 and incubated with FS patients and control sera. The bound autoantibodies were probed with monoclonal anti-human IgM. The figure shows anti-Dsg1 ELISA index values with (**a**) EDTA added and with (**b**) tunicamycin-treated Dsg1 plotted against the index values from the untreated samples. The 45° lines (solid) and the least-squares line (dashed) are shown. The paired *t*-test was significant: (**a**) $P=0.0042$ and (**b**) $P=0.044$.

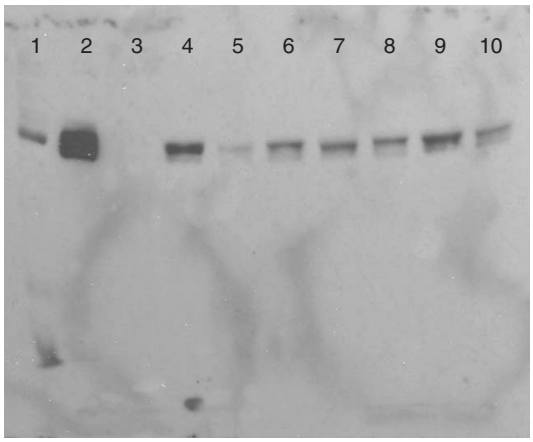


Figure 5. IgM immunoprecipitation of recombinant Dsg1.
Immunoprecipitation of recombinant Dsg1 by IgM (lanes 3–10) and IgG (lane 2) from the sera of FS patients (lanes 2, 7, 9, and 10), a PF patient (lane 4), a US normal donor (lane 3), and healthy donors from rural Brazilian Amerindian reservations (lanes 5, 6, and 8).All sera were tested at a 1:50 dilution, except the FS serum (lane 2) used to test for IgG anti-Dsg1 that was diluted at 1:200. Lane 1 is a molecular weight standard (66 kDa). It is clear that IgG (lane 2) and IgM autoantibodies recognize Dsg1 (lanes 4–10). Normal human serum (lane 3) was negative.

unimmunized individuals possess IgM antibodies to toxins, bacteria, and erythrocytes that comprise a population of polyreactive low-affinity natural antibodies (Manson *et al.*, 2005). These natural polyreactive IgM antibodies represent the first barrier against infection, eliminating bacteria by complement activation, thus bridging innate to adaptive immunity (Manson *et al.*, 2005). Many of these IgM natural antibodies recognize single or multiple self-antigens, and have been detected from early childhood throughout life (Mouthon *et al.*, 1996).

The IgM anti-Dsg1 autoimmune response observed in FS patients living in their native environment is distinctive, and represents a novel finding. The presence of IgM anti-Dsg1 autoantibodies in the sera of these patients was unrelated to the age, sex, or the clinical stage of the disease. It remains unknown if IgM, and pathogenic IgG and non-pathogenic IgG anti-Dsg1 autoantibodies may segregate according to the HLA of the individuals. A relatively high prevalence of IgM anti-Dsg1 autoantibodies was found in sera from both FS patients and clinically normal donors residing in rural settings in or near an endemic area. Patients with PF, other autoimmune bullous diseases, as well as healthy individuals from more urban settings did not demonstrate a significant IgM anti-Dsg1 response. Interestingly, the percentage of positive IgM anti-Dsg1 is low in FS patients, who temporarily resided in urban hospitals. It is likely that serum concentrations of IgM anti-Dsg1 autoantibodies from FS patients referred to metropolitan hospitals, away from their native environment, decrease due to the elimination of the environmental antigenic stimuli. We are prospectively evaluating FS patients who migrate out of their native environment to an urban hospital to test this hypothesis. We are also studying healthy Amerindians from LV or neighboring reservations, who have IgM anti-Dsg1 autoantibodies and migrate to urban centers away from their reservation, and non-Amerindian healthy new arrivals to these reservations. These studies may clarify the relationship of IgM anti-Dsg1 autoantibodies and exposure to endemic areas of FS. It is clear, however, that the humoral IgG and IgM autoantibody response in inhabitants of rural endemic regions of FS suggests strongly an environmental antigenic exposure, especially in LV, the active focus of the disease. Moreover, the IgM anti-Dsg1 response appears to distinguish FS patients living in their native environment from the non-endemic form of PF and other forms of pemphigus seen in other parts of the world.

It is well documented that pathogenic IgG anti-Dsg1 autoantibodies from PF and FS patients recognize conformational and Ca^{2+}-dependent epitopes on Dsg1 (Olague-Alcala and Diaz, 1993; Amagai *et al.*, 1995). We and others have also reported that IgG anti-Dsg1 antibodies bind peptide epitopes rather than carbohydrate moieties on the ectodomain of Dsg1 (Olague-Alcala and Diaz, 1993; Amagai *et al.*, 1995). In this study, we found that binding of IgM autoantibodies to Dsg1 is both calcium and N-glycosylation independent, suggesting that the epitopes recognized by IgM anti-Dsg1 autoantibodies are different from those bound by pathogenic IgG anti-Dsg1. These differences were also shown by ELISA inhibition studies, which showed that IgG anti-Dsg1 autoantibodies do not impair the binding of IgM anti-Dsg1 autoantibodies. Another interesting finding in this study is that IgM anti-Dsg1 reactivity was detected in healthy individuals as early as age 5. The cord sera of eight neonates did not contain IgM anti-Dsg1 autoantibodies but over 50% of sera from healthy donors between age of 5 and 20 from LV possessed IgM anti-Dsg1 autoantibodies. It is feasible that the ''time 0'' of the immunization process is the neonatal period and the production of IgM anti-Dsg1 begins gradually between this ''time 0'' and at the age of 5. There was no significant trend in IgM anti-Dsg1 autoantibody with age. However, the prevalence of IgG anti-Dsg1 autoantibodies gradually increased with age of the donors (Figure 3b). Notably, since the completion of the serological analysis for this study, one 19-year-old donor (IgG index value >80) has since developed clinically active disease.

In view of these findings, we propose that the IgM anti-Dsg1 autoantibody response represents the earliest event in the sensitization process in individuals living in these rural areas of Brazil. This event is followed by a gradual IgG response leading to FS. It is likely that the IgM response in individuals living in LV and other Amerindian reservations of the region arises from recurrent and persistent exposure to a putative environmental cross-reactive antigen(s) harbored in these regions. The exposure seems to occur in early childhood and continues throughout life, if the individuals remain in these regions. The resulting, likely low affinity IgM anti-Dsg1 autoantibodies, would be detected by highly sensitive ELISA assays but not by routine indirect IF studies. To further explore the reactivity with epithelial tissues by indirect IF, we selected a group of 20 sera from FS and PF patients, as well as healthy controls possessing IgM anti-Dsg1 autoantibodies. We found that all the 20 samples of sera did not bind the

epidermal cell surfaces by indirect IF. On the contrary, the sera of patients produced typical pemphigus IgG staining in these tissues. As shown in Figure 5, these IgM-positive sera bind Dsg1 by IP/IB techniques. Clinical disease may occur only in a small fraction of genetically predisposed individuals where the humoral immune response is switched from an IgM response to the production of pathogenic IgG anti-Dsg1 autoantibodies. Importantly, FS is common among poor outdoor young workers of both sexes and all races sharing the HLA DRB1*0404, DRB1*1402, or DRB1*1406 alleles (RR: 14) (Diaz *et al.*, 1989a, b; Moraes *et al.*, 1997).

The role of IgM autoantibodies in autoimmunity has recently become an area of great interest. Natural IgM autoantibodies against self-antigens have been reported in autoimmune diseases such as lupus erythematosus (Forger *et al.*, 2004; Ferreira *et al.*, 2005; Li *et al.*, 2005), autoimmune hemolytic anemia (Stahl and Sibrowski, 2005), and autoimmune thrombocytopenia (Stahl *et al.*, 2005). It is hypothesized that polyreactive IgM (Li *et al.*, 2005) or the IgG/IgM ratio of anti-dsDNA antibodies in systemic lupus erythematosus may modulate the disease and prognosis, especially in patients with nephritis (Forger *et al.*, 2004). Experimentally, passive transfer of monoclonal IgM anti-dsDNA autoantibodies blocks inflammatory organ damage in lupus-prone mice (NZB × NZW) F1 (Werwitzke *et al.*, 2005). Others have proposed that anti-Ro IgM autoantibodies in lupus may represent part of the natural IgM repertoire that leads to the production of pathogenic IgG autoantibodies by epitope spreading in genetically predisposed individuals (Ferreira *et al.*, 2005). The potential clinical and pathological significance of the IgM autoantibody response in the setting of FS is an area of ongoing investigation.

Several important issues remain to be addressed in future studies, such as the nature of the sensitizing environmental antigen(s), whether it is T-dependent or T-independent, and the role of self-Dsg1 in the production of these antibodies. Furthermore, identification of the Dsg1 epitopes recognized by IgM and IgG autoantibodies during the transition of the disease from preclinical to clinical stages will also be relevant. In conclusion, our studies suggest that IgM anti-Dsg1 autoantibodies are distinct markers of FS and may represent excellent probes to test environmental antigens linked to FS. FS is a fascinating model of a human organ-specific autoimmune disease, mediated by pathogenic IgG autoantibodies, where the immune response is linked to an environmental etiology. Elucidating the etiology of FS may provide insight into the pathogenesis of other human autoimmune diseases.

MATERIALS AND METHODS

Sources of sera from United States and Japan

The following groups of patients were tested: PF ($n=20$), mPV ($n=10$), mcPV ($n=10$), BP ($n=40$), and normal human sera ($n=55$) from North Carolina, USA. Most of these patients were receiving steroid or other immunosuppressive therapy at the time of serum collection. Many were in complete or partial clinical remission. Well-characterized sera from Japanese patients with mPV ($n=20$), mcPV ($n=20$), and PF ($n=20$) were obtained from Professor M. Amagai from Keio University in Tokyo.

Sources of sera from Brazilian patients and controls

Sera from FS patients were obtained from the LV reservation, state of Mato Grosso do Sul, Brazil ($n=31$). Twenty-three patients were on steroid therapy, and were in partial clinical remission at the time of collection. The remaining eight patients were asymptomatic. FS sera were also obtained from patients, most with extensive clinical disease, admitted to the following Brazilian hospitals: Hospital das Clinicas, Sao Paulo (Hospital-SP; $n=56$), Hospital de Doenças Tropicaes, Goiania (Hospital-GO; $n=42$), and Hospital Adventista do Penfigo, Campo Grande (Hospital-CG; $n=57$). Normal human sera were obtained from donors residing in four Amerindian reservations: LV ($n=103$), Bananal ($n=58$), Ipegue ($n=52$), and Agua Branca ($n=30$), all inhabited by Terena Amerindians settled in the state of Mato Grosso do Sul. Normal donors were also obtained from three urban Brazilian cities: Aquidauana, Mato Grosso do Sul ($n=27$), Campo Grande ($n=27$), and Sao Paulo ($n=27$). Figure 1 shows the locations of relevant Brazilian cities and Amerindian reservations.

Additional sources of sera

Additional sera were obtained from 99 young healthy donors, age 5–20 years, living in LV (34, age 5–10; 41, age 11–15; and 24, 16 and older). Studies carried out in this investigation were approved by the Institutional Review Boards of the University of North Carolina and the University of Sao Paulo, Brazil. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent. Also available to this investigation were eight sets of neonate/mother sera kept frozen since 1990 (Rocha-Alvarez *et al.*, 1992). These sera were part of a study evaluating the correlation of pemphigus autoantibodies in cord and mother's sera.

Production of baculovirus-expressed Dsg-1 (glycosylated and deglycosylated)

The ectodomain of Dsg-1 with a C-terminal His tag was produced in High Five™ insect cells by infection with the recombinant baculovirus stock of Dsg-1 (Ding *et al.*, 1999; Li *et al.*, 2003). To generate the deglycosylated Dsg1, tunicamycin (Sigma, St Louis, MO) was added to the culture medium (0.5 $\mu g\,ml^{-1}$) at the time of infection. The tunicamycin-treated (deglycosylated) and untreated (glycosylated) recombinant Dsg1 were purified by nickel affinity chromatography as described (Ding *et al.*, 1999; Li *et al.*, 2003) and used for ELISA assay.

Anti-Dsg1 IgM and anti-Dsg1 IgA autoantibodies by IgG ELISA assays

ELISA plates were coated with 200 ng per 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 (Tris-buffered saline/Ca^{2+}/Tween-20), the plate was blocked with 1% BSA in Tris-buffered saline/Ca^{2+}/Tween-20 at room temperature for 1 hour. The plate was then incubated with duplicate 1:100 dilutions of serum samples for 1 hour at room temperature. Following wash, the plate was incubated with a 1:1000 dilution of horseradish peroxidase-labeled mouse anti-human IgM or with 1:2000 dilution of horseradish peroxidase-conjugated mouse anti-human IgG (Zymed, San Francisco, CA) (Warren *et al.*, 2000, 2003). The assays produced a linear reaction in the range of 6.25–800 ng of purified IgM or IgG. Similar

procedures were followed to set the Dsg1 IgA ELISA, using Dsg1 immobilized on Ni-coated microtiter plates and monoclonal anti-IgA antibodies (Zymed). A positive serum from a patient with IgA pemphigus (showing a titer of IgA anti-epidermal cell surface autoantibodies of 1:640 by indirect IF) was used as a positive control. Positive, negative, and test sera were tested at a 1:100 dilution. Since the IgA pemphigus serum used as a positive control exhibited marginal optical density (OD) readings at 490 nm above background, it was not possible to determine reliable index values, hence OD_{490} values were used to evaluate the reactivity of test sera. A single FS serum from an LV patient who produced consistent and reproducible positive index values for IgM anti-Dsg1 autoantibodies was selected as a positive control throughout. Similarly a normal human serum from USA was used as a negative control. Results were expressed as index value units as reported by Amagai *et al.* (1999). The index value was defined in terms of OD as follows:

$$\text{index value} = \frac{(\text{test sample OD}) - (\text{negative control OD})}{(\text{positive control OD}) - (\text{negative control OD})} \times 100$$

The cutoffs for positive IgM and IgG values were determined by testing 31 FS patients from LV (for IgM), 20 PF patients from Japan (for IgG), and 50 normal human sera from United States. The index values of IgM anti-Dsg1 produced by FS sera and normal human sera were utilized to plot a receiver operator characteristic curve (see Figure S4). We determined that index values of IgM anti-Dsg1 autoantibodies above 50 provide 58% sensitivity and 96% specificity. Additionally, index values of IgG anti-Dsg1 autoantibodies above 10 were associated with a 100% observed sensitivity and specificity (see Figure S5). The cutoff of 50 for IgM and 10 for IgG were used throughout these studies. The percentages of positive samples for IgM and IgG anti-Dsg1 autoantibodies were determined for each group tested. The distribution of individual samples in each group was also expressed as box plots (see below).

For calcium effect study, 5 mM EDTA was added to Tris-buffered saline without Ca^{2+}, and the buffer was used in the ELISA. Reproducibility of the Dsg1 ELISA for IgM and IgG was performed by two independent investigators testing a set of 48 sera selected and coded by a third individual. The results demonstrated a nearly perfect linear relationship with correlation coefficient of 0.977 ($P<0.001$). Readings by one reader tended to be greater in magnitude by about 11%. Using either reader's results would produce nearly identical results for the hypotheses of interest in this report.

To test for competitive inhibition of IgG and IgM anti-Dsg1 autoantibodies for the binding sites of immobilized Dsg1 on the ELISA microtiter wells, the following experiments were performed: a set of FS sera with a high-titer IgG anti-Dgs1 and low IgM anti-Dsg1 were selected and were tested at 1:100, 1:200, 1:400, and 1:800 dilutions. Index values were determined for each dilution. Additionally, microtiter wells coated with Dsg1 were preincubated with a high-titer IgG anti-Dsg1 serum at a dilution of 1:100, then tested using a 1:100 dilution of high IgM anti-Dsg1 sera. The remainder of the ELISA procedure was carried out as described above.

Indirect IF and immunoprecipitation/immunoblotting assays

Indirect IF techniques were carried out as reported previously (Rock *et al.*, 1989). Briefly, human skin and monkey esophagus cryosections were incubated with a 1:20 and 1:40 dilutions of test sera. Positive sera were titrated to the end point. Human IgG antibodies were detected using FITC-labelled goat anti-human IgG serum (MP Biomedicals LLC, Solon, OH) and human IgM autoantibodies with murine monoclonal anti-human IgM antibodies (Southern Biotech, Birmingham, AL) and FITC-labelled sheep anti-mouse IgG (MP Biomedicals LLC).

Immunoprecipitation followed by immunoblotting was carried out as described previously (Ding *et al.*, 1999; Li *et al.*, 2003). Briefly, the sera from patients and controls were incubated with recombinant Dsg1 at 4°C overnight. IgM anti-Dsg1 autoantibodies were immunoprecipitated using goat anti-human IgM (U-chain specific)-Agarose coated beads (Sigma-Aldridge, St Louis, MO). IgG anti-Dsg1 autoantibodies were immunoprecipitated using recombinant Staph protein G-coated Sepharose 4B beads (Invitrogen, Carlsbad, CA). Immunoprecipitated Dsg1 was extracted with 1% SDS, run on 10% SDS–PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA), and finally probed with a 1:2000 dilution of horseradish peroxidase-labeled monoclonal anti-HIS antibodies (Qiagen, Valencia, CA). Specific bands were demonstrated by chemiluminescense (GE Healthcare Limited, Piscataway, NJ).

Statistical analysis

The distribution of index values of each sample for each group was determined and a box plot constructed. The box plot of each group was analyzed for significance and trends. Differences among groups were analyzed using one-way analysis of variance. Further analyses used non-parametric one-way analysis (Mann–Whitney and VDW) and Winsorization. Analyses were carried out in SAS, version 9.1, and R, version 2.2. Linear regression was used for estimation of IgM and IgG trends with age. For paired data, as in Figure 4, the pair *t*-test was used. The Supplementary Material includes box plots of IgM and IgG in the different groups.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This work was supported in part by US Public Health Service Grants R01-AR30281, RO1-AR32599, and T32 AR07369 awarded to Dr Luis A. Diaz, and KO1 AR052109 and RO3 AR053313 awarded to Dr Ning Li. Dr David Dasher is a dermatology research fellow supported by the T32 AR07369 training grant. Dr Masayuki Amagai from Keio University kindly provided well-characterized pemphigus sera. We thank the collaboration and support of the Terena Amerindian people of Limao Verde, Bananal, Ipegue, and Agua Branca, Brazil.

SUPPLEMENTARY MATERIAL

Figure S1. Box plot representation of IgM and IgG anti-Dsg1 reactivity of sera from patients with FS and PF.

Figure S2. Box plot representation of IgM and IgG anti-Dsg1 reactivity of sera from healthy control individuals from rural and urban sites.

Figure S3. Scatter representation of IgM and IgG anti-Dsg1 reactivity of sera from healthy donors age 5–20.

Figure S4. Receiver operator characteristic curve of IgM anti-Dsg1 index values of FS sera ($N=31$) and NHS ($N=55$) and cut point determination of sensitivity and specificity.

Figure S5. Receiver operator characteristic curve of IgG anti-Dsg1 index values of Japanese PF sera ($N=20$) and NHS ($N=50$) and cut point determination of sensitivity and specificity.

REFERENCES

Abbas AK, Lichtman AH (2005) B cell activation and antibody production. In: *Cellular and molecular immunology* (Abbas AK, Lichtman AH, eds), 5th ed. Philadelphia, PA: Elsevier Saunders, 190–1

Amagai M, Ishii K, Hashimoto T, Gamou S, Shimizu N, Nishikawa T (1995) Conformational epitopes of pemphigus antigens (Dsg1 and Dsg3) are calcium dependent and glycosylation independent. *J Invest Dermatol* 105:243–7

Amagai M, Klaus-Kovtun V, Stanley JR (1991) Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 67:869–77

Amagai M, Tsunoda K, Zillikens D, Nagai T, Nishikawa T (1999) The clinical phenotype of pemphigus is defined by the anti-desmoglein autoantibody profile. *J Am Acad Dermatol* 40:167–70

Aoki V, Millikan RC, Rivitti EA, Hans-Filho G, Eaton DP, Warren SJ *et al.* (2004) Environmental risk factors in endemic pemphigus foliaceus (fogo selvagem). *J Invest Dermatol* 9:34–40

Cermakova Z, Ryskova O, Honegr K, Cermakova E, Hanovcova I (2005) Diagnosis of Lyme borreliosis using enzyme immunoanalysis. *Med Sci Monit* 11:121–5

Chu CM (2006) Immunoglobulin class M anti-hepatitis B core antigen for serodiagnois of acute hepatitis: pitfalls and recommendations. *J Gastroenterol Hepatol* 21:789–91

Diaz LA, Arteaga LA, Hilario-Vargas J, Valenzuela JG, Li N, Warren SJ *et al.* (2004) Anti-desmoglein-1 antibodies in onchocerciasis, leishmaniasis and Chagas disease suggest a possible etiological link to fogo selvagem. *J Invest Dermatol* 123:1045–51

Diaz LA, Sampaio SAP, Rivitti EA, Martins CR, Cunha PR, Lombardi C *et al.* (1989a) Endemic pemphigus foliaceus (fogo selvagem): I. Clinical features and immunopathology. *J Am Acad Dermatol* 20:657–69

Diaz LA, Sampaio SAP, Rivitti EA, Martins CR, Cunha PR, Lombardi C *et al.* (1989b) Endemic pemphigus foliaceus (fogo selvagem): II. Current and historic epidemiologic studies. *J Invest Dermatol* 92:4–12

Ding X, Aoki V, Mascaro JM Jr, Lopez-Swiderski A, Diaz LA, Fairley JA (1997) Mucosal and mucocutaneous (generalized) pemphigus vulgaris show distinct autoantibody profiles. *J Invest Dermatol* 109:592–6

Ding X, Diaz LA, Fairley JA, Giudice GJ, Liu Z (1999) The anti-desmoglein 1 autoantibodies in pemphigus vulgaris sera are pathogenic. *J Invest Dermatol* 112:739–43

Empinotti JC, Aoki V, Filgueira A, Sampaio SA, Rivitti EA, Sanches JA Jr *et al.* (2006) Clinical and serological follow-up studies of endemic pemphigus foliaceus (fogo selvagem) in Western Parana, Brazil (1988–2003). *Br J Dermatol* 155:446–50

Ferreira R, Barreto M, Santos E, Pereira C, Martins B, Andreia R *et al.* (2005) Heritable factors shape natural human IgM reactivity to Ro60/SS-A and may predispose for SLE-associated IgG anti-Ro and anti-La autoantibody production. *J Autoimmun* 25:155–63

Forger F, Matthias T, Oppermann M, Becker H, Helme K (2004) Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis. *Lupus* 13:36–44

Hamkar R, Jalilvand S, Mokhtari-Azad T, Nouri Jelyani K, Dahi-Far H, Soleimanjahi H *et al.* (2005) Assessment of IgM enzyme immunoassay and IgG avidity assay for distinguishing between primary and secondary immune response to rubella vaccine. *J Virol Methods* 130:59–65

Hans-Filho G, dos Santos V, Katayama JH, Aoki V, Rivitti EA, Sampaio SA *et al.* (1996) An active focus of high prevalence of fogo selvagem on an Amerindian reservation in Brazil. *J Invest Dermatol* 107:68–75

Hilario-Vargas J, Dasher DA, Li N, Aoki V, Hans-Filho G, dos Santos V *et al.* (2006) Prevalence of anti-desmoglein-3 antibodies in Endemic regions of fogo selvagem in Brazil. *J Invest Dermatol* 126:2044–8

Li N, Aoki V, Hans-Filho G, Rivitti EA, Diaz LA (2003) The role of intramolecular epitope spreading in the pathogenesis of endemic pemphigus foliaceus (fogo selvagem). *J Exp Med* 197:1501–10

Li QZ, Xie C, Wu T, Mackay M, Aranow C, Putterman C *et al.* (2005) Identification of autoantibody clusters that best predict lupus disease activity using glomerular proteome arrays. *J Clin Invest* 115: 3428–39

Liu Z, Diaz LA (2001) Bullous pemphigoid: end of the century overview. *J Dermatol* 28:647–50

Manson JJ, Mauri C, Ehrenstein MR (2005) Natural serum IgM maintains immunological homeostasis and prevents autoimmunity. *Springer Semin Immunopathol* 26:425–32

Montoya JG (2002) Laboratory diagnosis of toxoplasma gondii infection and toxoplasmosis. *J Infect Dis* 185(Suppl 1):S73–82

Moraes ME, Fernandez-Viña M, Lazaro A, Diaz LA, Filho GH, Friedman H *et al.* (1997) An epitope in the third hypervariable region of the DRB1 gene is involved in the susceptibility to endemic pemphigus foliaceus (fogo selvagem) in three different Brazilian populations. *Tissue Antigens* 49:35–40

Mouthon L, Lacroix-Desmazes S, Nobrega A, Barreau C, Coutinho A, Kazatchkine MD (1996) The self-reactive antibody repertoire of normal human serum IgM is acquired in early childhood and remains conserved throughout life. *Scand J Immunol* 44:243–51

Obel N, Hoier-Madsen M, Kangro H (1996) Serological and clinical findings in patients with serological evidence of reactivated Epstein–Barr virus infection. *APMIS* 104:424–8

Olague-Alcala M, Diaz LA (1993) The epitopes on bovine pemphigus foliaceus antigen are calcium dependent and located on the peptide backbone of this glycoprotein. *Chron Dermat* 2:189–209

Reis MM, Tessaro MM, D'Azevedo PA (2006) Toxoplasma-IgM and IgG-avidity in single samples from areas with a high infection rate can determine the risk of mother-to-child transmission. *Rev Inst Med Trop Sao Paulo* 48:93–8

Rocha-Alvarez R, Friedman H, Campbell I, Souza-Aguiar L, Martins-Castro R, Diaz LA (1992) Pregnant women with endemic pemphigus foliaceus (fogo selvagem) give birth to disease-free babies. *J Invest Dermatol* 99:78–82

Rocha-Alvarez R, Ortega-Loayza A, Friedman H, Campbell H, Aoki V, Rivitti EA *et al.* (2007) Endemic pemphigus vulgaris. *Arch Dermatol* 143:895–9

Rock B, Martins CR, Theofilopoulos AN, Balderas RS, Anhalt GJ, Labib RS *et al.* (1989) The pathogenic effect of IgG4 autoantibodies in endemic pemphigus foliaceus (fogo selvagem). *N Engl J Med* 320:1463–9

Squiquera HL, Diaz LA, Sampaio SAP, Rivitti EA, Martins CR, Cunha PR *et al.* (1988) Serological abnormalities in patients with endemic pemphigus foliaceus (fogo selvagem), their relatives and normal donors from endemic and non-endemic areas of Brazil. *J Invest Dermatol* 91: 189–91

Stahl D, Hoemberg M, Cassens U, Pachmann U, Sibrowski W (2005) Evidence that human autoimmune thrombocytopenia mediated by both immunoglobulin isotypes IgM and IgG is an independent disease entity. *Eur J Haematol* 75:318–27

Stahl D, Sibrowski W (2005) Warm autoimmune hemolytic anemia is an IgM–IgG immune complex disease. *J Autoimmunity* 25:272–82

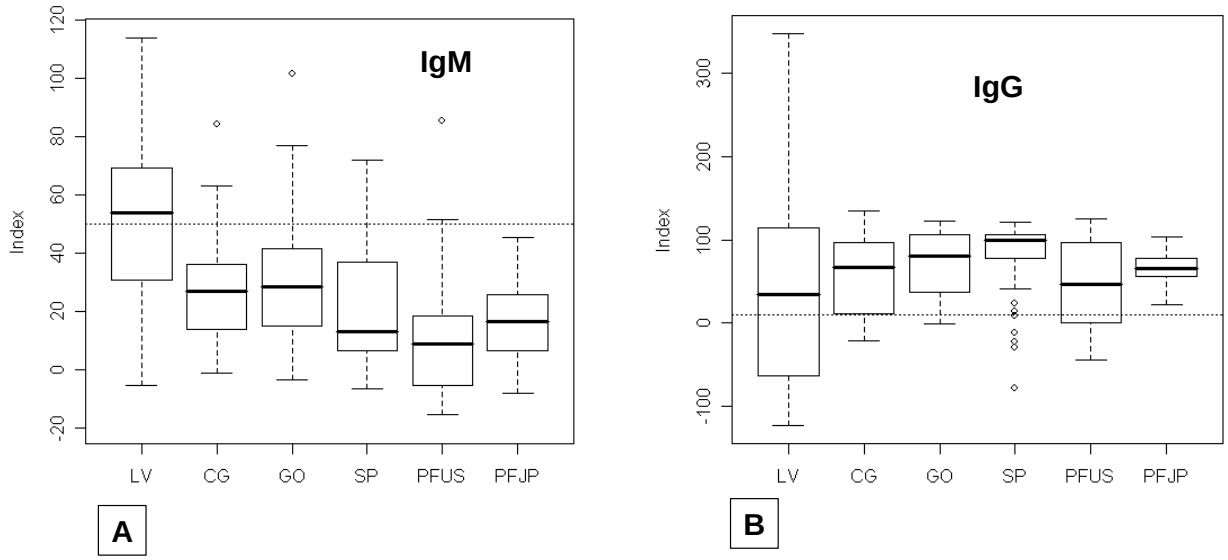
Stanley JR, Klaus-Kovtun V, Sampaio SAP (1986) Antigenic specificity of fogo selvagem autoantibodies is similar to North American pemphigus foliaceus and distinct from pemphigus vulgaris. *J Invest Dermatol* 87:197–201

Warren SJP, Arteaga LA, Rivitti EA, Aoki V, Hans-Filho G, Qaqish BF *et al.* (2003) The role of subclass switching in the pathogenesis of endemic pemphigus foliaceus. *J Invest Dermatol* 120:104–8

Warren SJP, Lin MS, Giudice GJ, Hoffman RG, Hans-Filho G, Aoki V *et al.* (2000) The prevalence of antibodies against desmoglein 1 in endemic pemphigus foliaceus in Brazil. *N Engl J Med* 343:23–30

Werwitzke S, Trick D, Kamino K, Matthias T, Kniesch K, Schlegelberger B *et al.* (2005) Inhibition of lupus disease by anti-double-stranded DNA antibodies of the IgM isotype in the (NZB × NZW)F1 mouse. *Arthritis Rheum* 52:3629–38

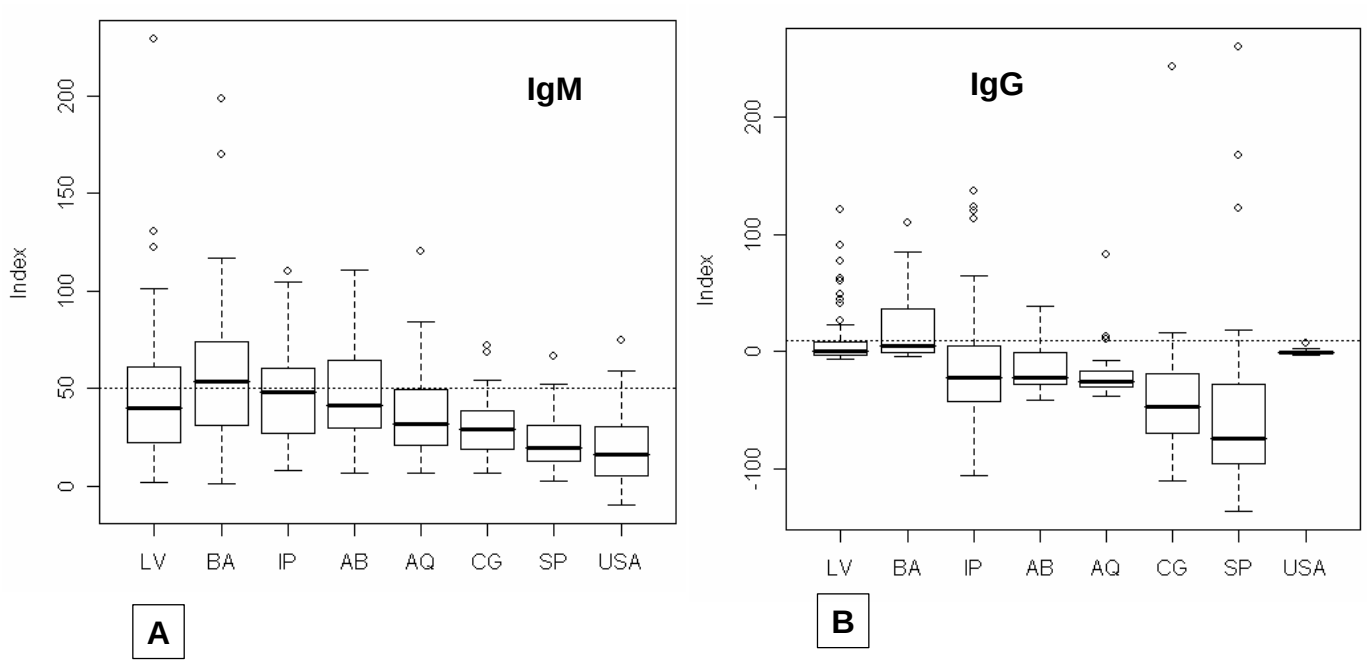
Anti-Dsg1 IgM and IgG Autoantibodies in FS and PF Donors



Supplementary Figure 1

Supplementary Figure 1: Boxplot representation of IgM and IgG anti-Dsg1 reactivity of sera from patients with FS and PF. Panel A (left) shows the index values produced by IgM autoantibodies of sera of each group (LV: Limao Verde, CG: Campo Grande, GO: Goiania, SP: Sao Paulo, PFUS: pemphigus foliaceus from USA and PFJP: pemphigus foliaceus from Japan) and Panel B (right) the IgG index values. The horizontal broken lines in each panel represent the cutoff for IgM (50 units; 58% sensitivity and 96% specificity) and IgG (10 units; 100% observed sensitivity and specificity) ELISA assays.

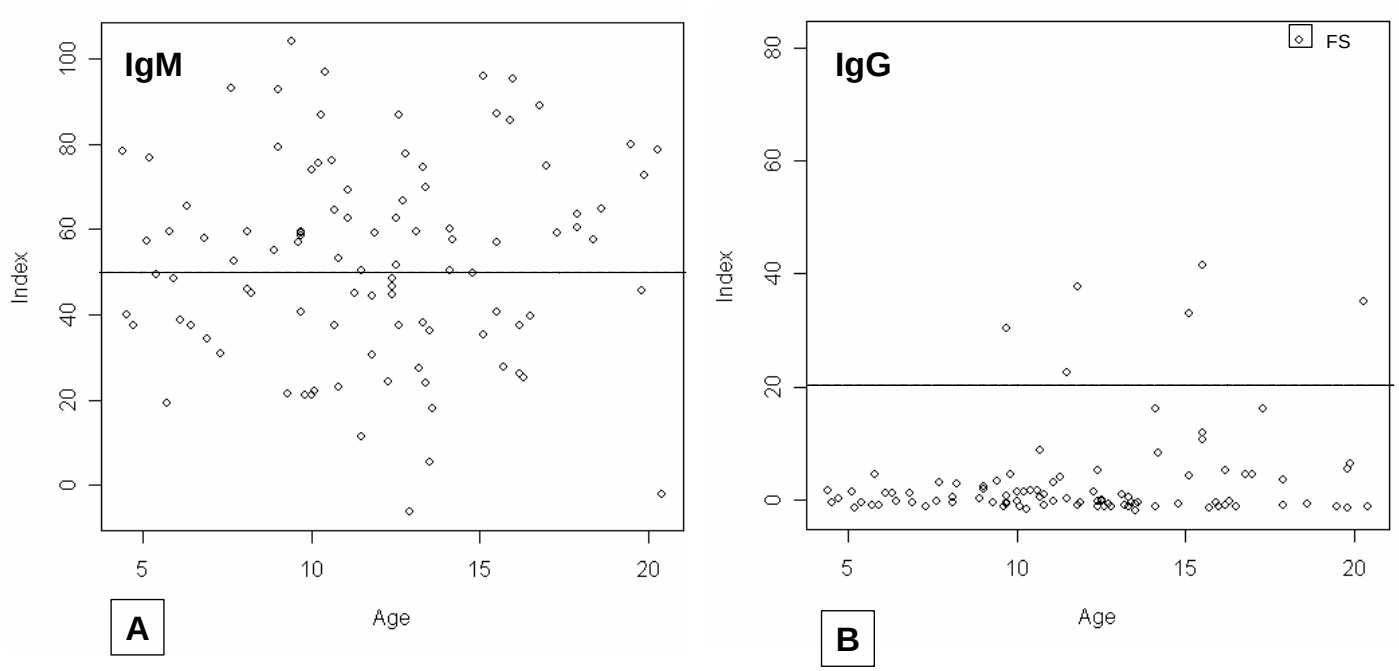
Anti-Dsg1 IgM and IgG Autoantibodies in Healthy Donors from Rural and Urban Sites



Supplementary Figure 2

Supplementary Figure 2: Boxplot representation of IgM and IgG anti-Dsg1 reactivity of sera from healthy control individuals from rural and urban sites. Panel A (left) shows the index values produced by IgM autoantibodies of sera of each group (LV:Limao Verde, BA:Bananal, IP: Ipegue, AB: Agua Branca, AQ: Aquidauana, CG: Campo Grande, SP: Sao Paulo, USA: United States of America) and Panel B (right) the IgG index values of the same subjects. The horizontal broken lines in each panel represents the cutoff for IgM (50 units; 58% sensitivity and 96% specificity) and IgG (10 units; 100% observed sensitivity and specificity).

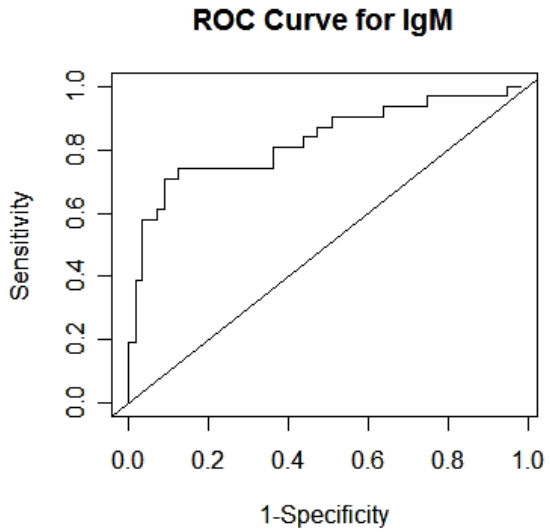
Anti-Dsg1 IgM and IgG Autoantibodies in Healthy Donors (Ages 5-20)



Supplementary Figure 3

Supplementary Figure 3: Scatter representation of IgM and IgG anti-Dsg1 reactivity of sera from healthy donors ages 5 to 20. The index values for IgM anti-Dsg1 (Panel A) and IgG anti-Dsg1 (Panel B) are plotted against age in years. The donor showing the highest index value for IgG anti-Dsg1 (encircled and labeled FS) developed FS during the course of this study.

ROC curve of IgM anti-Dsg1 Index Values of FS sera (N=31) and NHS (N=55) and Cutpoint Determination of Sensitivity and Specificity



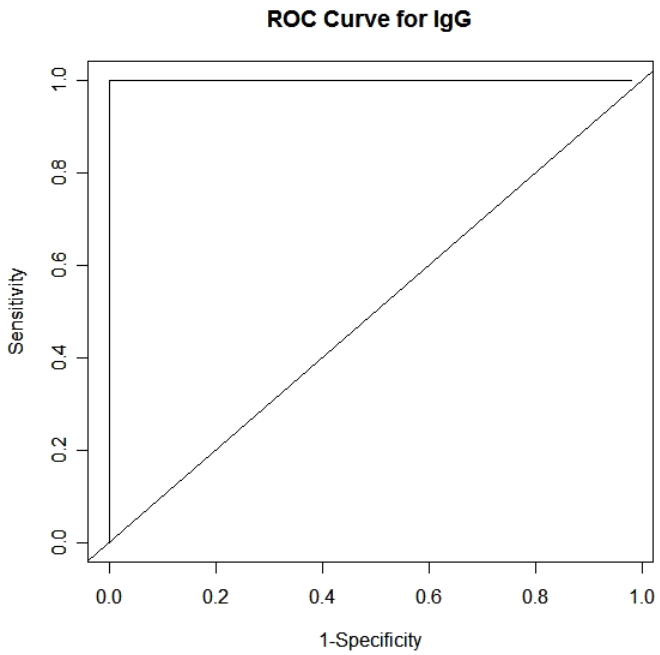
Sensitivity and Specificity: IgM anti-Dsg1 ELISA (FS vs Healthy control sera)

	Cutpoint	Sensitivity	Specificity
#1	-9.42	1.00	0.02
#2	-7.03	1.00	0.04
#3	-6.09	1.00	0.05
#4	-5.56	0.97	0.05
#5	-5.07	0.97	0.07
#6	-3.79	0.97	0.09
#7	-2.70	0.97	0.11
#8	-2.03	0.97	0.13
#9	-1.31	0.97	0.15
#10	-1.09	0.97	0.16
#11	1.74	0.97	0.18
#12	1.88	0.97	0.20
#13	2.75	0.97	0.22
#14	3.48	0.97	0.24
#15	5.07	0.97	0.25
#16	5.37	0.94	0.25
#17	5.87	0.94	0.27
#18	6.96	0.94	0.29
#19	8.04	0.94	0.31
#20	8.42	0.94	0.33
#21	11.16	0.94	0.35
#22	11.81	0.94	0.36
#23	12.80	0.91	0.36
#24	13.12	0.91	0.38
#25	13.26	0.91	0.40
#26	13.99	0.91	0.42

#27	14.20	0.91	0.44
#28	14.93	0.91	0.45
#29	15.05	0.91	0.47
#30	16.30	0.91	0.49
#31	17.94	0.88	0.49
#32	18.33	0.88	0.51
#33	18.70	0.88	0.53
#34	18.73	0.84	0.53
#35	19.57	0.84	0.55
#36	20.72	0.84	0.56
#37	21.94	0.81	0.56
#38	22.46	0.81	0.58
#39	22.92	0.81	0.60
#40	23.91	0.81	0.62
#41	24.06	0.81	0.64
#42	24.24	0.78	0.64
#43	24.70	0.75	0.64
#44	25.22	0.75	0.65
#45	25.29	0.75	0.67
#46	26.45	0.75	0.69
#47	30.00	0.75	0.71
#48	30.14	0.75	0.73
#49	30.29	0.75	0.76
#50	32.83	0.75	0.78
#51	33.04	0.75	0.80
#52	33.55	0.75	0.82
#53	33.91	0.75	0.84
#54	34.86	0.75	0.85
#55	35.00	0.75	0.87
#56	37.05	0.72	0.87
#57	38.12	0.72	0.89
#58	45.22	0.72	0.91
#59	46.17	0.69	0.91
#60	46.24	0.66	0.91
#61	47.65	0.63	0.91
#62	47.81	0.63	0.93
#63	48.17	0.59	0.93
#64	48.51	0.59	0.95
#65*	49.13	0.59	0.96
#66	51.55	0.56	0.96
#67	52.32	0.53	0.96
#68	53.76	0.50	0.96
#69	54.24	0.47	0.96
#70	56.32	0.44	0.96
#71	56.75	0.41	0.96
#72	58.77	0.41	0.98
#73	60.32	0.38	0.98
#74	61.77	0.34	0.98
#75	65.02	0.31	0.98
#76	67.30	0.28	0.98
#77	70.93	0.25	0.98

#78	73.50	0.22	0.98
#79	74.57	0.22	1.00
#80	75.11	0.19	1.00
#81	77.01	0.16	1.00
#82	79.29	0.13	1.00
#83	81.19	0.09	1.00
#84	98.36	0.06	1.00
#85	107.43	0.03	1.00
#86	113.90	0.00	1.00
* Cutpoint = or > 50 is considered positive serum			

ROC curve of IgG anti-Dsg1 Index Values of Japanese PF sera (N=20) and NHS (N=50) and Cutpoint Determination of Sensitivity and Specificity



Anti-Dsg1 IgG ELISA-Index Values

	cutpoint	sensitivity	specificity
#1	-2.51	1	0.02
#2	-2.36	1	0.04
#3	-2.29	1	0.06
#4	-2.25	1	0.08
#5	-2.19	1	0.1
#6	-2.17	1	0.14
#7	-2.11	1	0.16
#8	-2.06	1	0.18
#9	-2.03	1	0.2
#10	-2.02	1	0.24
#11	-1.97	1	0.26
#12	-1.96	1	0.28
#13	-1.94	1	0.3
#14	-1.89	1	0.32
#15	-1.87	1	0.34
#16	-1.79	1	0.36
#17	-1.62	1	0.38
#18	-1.47	1	0.4
#19	-1.45	1	0.42
#20	-1.42	1	0.44
#21	-1.39	1	0.46
#22	-1.35	1	0.48
#23	-1.18	1	0.5

#24	-0.99	1	0.54
#25	-0.82	1	0.56
#26	-0.61	1	0.58
#27	-0.57	1	0.6
#28	-0.43	1	0.62
#29	-0.40	1	0.64
#30	-0.29	1	0.68
#31	-0.23	1	0.7
#32	-0.17	1	0.72
#33	-0.13	1	0.74
#34	-0.06	1	0.76
#35	0.13	1	0.78
#36	0.13	1	0.8
#37	0.19	1	0.82
#38	0.41	1	0.84
#39	0.58	1	0.86
#40	0.95	1	0.88
#41	1.83	1	0.9
#42	2.25	1	0.92
#43	2.32	1	0.94
#44	2.32	1	0.96
#45	2.49	1	0.98
#46*	7.70	1	1
#47	35.10	0.95	1
#48	39.39	0.9	1
#49	50.16	0.85	1
#50	58.01	0.8	1
#51	85.72	0.75	1
#52	86.65	0.7	1
#53	94.06	0.65	1
#54	95.89	0.6	1
#55	98.03	0.55	1
#56	100.64	0.5	1
#57	101.71	0.45	1
#58	111.03	0.4	1
#59	113.03	0.35	1
#60	116.85	0.3	1
#61	120.24	0.25	1
#62	121.17	0.2	1
#63	127.02	0.15	1
#64	147.47	0.1	1
#65	160.15	0.05	1
#66**	160.70	0	1
Cutpoint = or > 10 is considered positive serum			
**Four normal human sera produce similar values			