Development of an IgG4-Based Predictor of Endemic Pemphigus Foliaceus (Fogo Selvagem)

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Fogo selvagem (FS) is mediated by pathogenic, predominantly IgG4, anti-desmoglein 1 (Dsg1) autoantibodies and is endemic in Limao Verde, Brazil. IgG and IgG subclass autoantibodies were tested in a sample of 214 FS patients and 261 healthy controls by Dsg1 ELISA. For model selection, the sample was randomly divided into training (50%), validation (25%), and test (25%) sets. Using the training and validation sets, IgG4 was chosen as the best predictor of FS, with index values above 6.43 classified as FS. Using the test set, IgG4 has sensitivity of 92% (95% confidence interval (95% CI): 82–95%), specificity of 97% (95% CI: 89–100%), and area under the curve of 0.97 (95% CI: 0.94–1.00). The IgG4 positive predictive value (PPV) in Limao Verde (3% FS prevalence) was 49%. The sensitivity, specificity, and PPV of IgG anti-Dsg1 were 87, 91, and 23%, respectively. The IgG4-based classifier was validated by testing 11 FS patients before and after clinical disease and 60 Japanese pemphigus foliaceus patients. It classified 21 of 96 normal individuals from a Limao Verde cohort as having FS serology. On the basis of its PPV, half of the 21 individuals may currently have preclinical FS and could develop clinical disease in the future. Identifying individuals during preclinical FS will enhance our ability to identify the etiological agent(s) triggering FS.

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Journal of Investigative Dermatology (2009) **129,** 110–118; doi:10.1038/jid.2008.189; published online 14 August 2008

INTRODUCTION

Both non-endemic pemphigus foliaceus (PF) and its endemic form (fogo selvagem (FS)) are characterized by subcorneal epidermal blisters and pathogenic IgG anti-desmoglein 1 (Dsg1) autoantibodies (Beutner and Jordon, 1964; Roscoe *et al.*, 1985; Stanley *et al.*, 1986; Diaz *et al.*, 1989a). FS patients usually live in impoverished rural areas of certain states of Brazil where the disease is endemic (Vieira, 1937; Diaz *et al.*, 1989b). Strikingly, the prevalence of FS in some states, such as Sao Paulo (Diaz *et al.*, 1989b) and Parana (Empinotti *et al.*, 2006), has decreased dramatically in recent years. An endemic form of PF has also been described in Colombia and Tunisia (Robledo *et al.*, 1988; Morini *et al.*, 1993; Abreu-Velez *et al.*, 2003). FS exhibits a strong association with the HLA-DRB1*0102, HLA-DRB1*0404, and HLA-DRB1*1402 alleles ($P<0.005$, Relative risk: 14) and affects people of many races and ethnic backgrounds (Moraes *et al.*, 1997). It has been hypothesized that a local environmental agent(s), acting on genetically predisposed individuals, triggers a crossreactive anti-Dsg1 antibody response that leads to FS (Diaz *et al.*, 1989b). Recent studies suggest that exposure to hematophagous insect bites is a risk factor for FS (Lombardi *et al.*, 1992; Aoki *et al.*, 2004; Diaz *et al.*, 2004).

IgG autoantibodies in FS are isotype-restricted, and the bulk of pathogenic anti-Dsg1 autoantibodies are predominantly IgG4 (Rock *et al.*, 1989; Santos *et al.*, 2001; Warren *et al.*, 2003). In fact, a recent study showed that progression from preclinical to clinical stage of the disease is associated with a dramatic rise in IgG4 anti-Dsg1 autoantibodies as determined by ELISA assays (Warren *et al.*, 2003). Restriction of IgG4 antibody response in humans has been reported in patients with parasitosis (Kurniawan *et al.*, 1993), individuals undergoing hyposensitization therapy for allergies (Larche *et al.*, 2006; Rossi *et al.*, 2007), individuals exposed to bee stings (Aalberse *et al.*, 1983), and patients with autoimmune pancreatitis (Hamano *et al.*, 2001). IgG4 restriction of the autoimmune response has also been reported in other autoimmune blistering diseases, although there is limited information about their pathogenic role in skin disease (Sitaru *et al.*, 2007).

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Abbreviations: AUC, area under the curve; CI, confidence interval; Dsg, desmoglein; FS, fogo selvagem; mcPV, mucocutaneous PV; NPV, negative predictive value; PF, pemphigus foliaceus; PPV, positive predictive value; PV, pemphigus vulgaris

Received 10 January 2008; revised 29 April 2008; accepted 26 May 2008; published online 14 August 2008

Anti-Dsg1 autoantibodies in PF and FS are routinely detected by indirect immunofluorescence (IF) techniques and are important diagnostic markers of these diseases. The use of PF and FS autoantibodies as predictors of disease has been limited because of the rarity of these diseases and the limitations of the indirect immunofluorescence assays. The application of ELISA techniques using recombinant Dsg1 has improved diagnostic accuracy (Amagai *et al.*, 1999) and made it possible to test large numbers of individuals living in communities where FS is endemic (Hans-Filho *et al.*, 1996). For example, we have identified a new focus of FS in Brazil, the Amerindian Terena reservation of Limao Verde, where the prevalence of the disease is ~3% (Hans-Filho *et al.*, 1996). We have reported that 30 of 31 patients along with 55% of normal participants ($n = 93$) living in this reservation possessed anti-Dsg1 antibodies (Warren *et al.*, 2000; Diaz *et al.*, 2008). We have followed this reservation for the past 14 years and documented the progression of FS from the preclinical stage to disease in 11 cases. Serologically, these patients increased their titers of anti-Dsg1 autoantibodies at the onset of their skin disease (Warren *et al.*, 2000), and importantly, the autoantibodies during the preclinical stage bind the extracellular 5 (EC5) domain of Dsg1, whereas the pathogenic autoantibodies recognized epitopes located on the EC1–2 domain of the molecule during the clinical stage (Li *et al.*, 2003).

In an effort to identify early sensitive and specific serological markers of FS in healthy individuals, we tested IgG and the IgG subclass anti-Dsg1 autoantibody response by ELISA in a large number of patients and normal donors. A rigorous statistical analysis of the data generated has permitted us to develop an IgG4 classifier/predictor that separates donors into one group showing immunological features of FS and another group with features of healthy donors. The IgG4 classifier is highly sensitive (92%) and specific (97%). In a population with the prevalence of 3%, that is Limao Verde, this classifier has positive predictive value (PPV) of 49% and negative predictive value (NPV) of 99.7%. The sensitivity, specificity, and PPV of IgG anti-Dsg1 were 87, 91, and 23%, respectively, all lower than the corresponding values for IgG4. This instrument has been validated in two patient populations; a group of 60 Japanese patients with PF and pemphigus vulgaris (PV) and a group of 11 FS patients from Limao Verde where preclinical stage sera were available. The use of this classifier tool may facilitate the identification of individuals during the preclinical stage of FS, thus enhancing the chances of disclosing the etiological agent(s) triggering this human autoimmune disease. As IgG anti-Dsg1 autoantibodies are detected in a large number of normal individuals from endemic areas of FS (Warren *et al.*, 2000, 2003; Diaz *et al.*, 2008), the high specificity of the IgG4 anti-Dsg1 classifier will outperform the total IgG assays when used in these seroepidemiological studies.

RESULTS

Development of the predictor

We used data on sera from 214 FS cases (45%) and 261 healthy controls (non-cases) (55%). The results of the IgG subclass and total IgG anti-Dsg1 ELISA of FS cases and controls were expressed as index values. Table 1 gives descriptive statistics of IgG index values.

Table 1. Descriptive statistics for IgG and IgG subclass index values

	FS cases					Healthy controls				
	N	Mean	Median	IQR	SD	*N*	Mean	Median	IQR	SD
IgG1	214	232.1	73.2	233.1	419.6	261	50.3	0	2.4	206.1
IgG2	214	50.7	0.3	49.9	113.7	261	26.5	2.5	29.7	59.6
IgG3	214	10.6	5.5	9.6	15.0	261	4.5	1.7	3.5	9.2
IgG4	214	76.5	69.0	79.1	53.7	261	1.6	0	1.2	4.6
IgG	209	79.6	88.0	35.8	32.5	250	2.4	0	0	11.7

FS, fogo selvagem; IQR, interquartile range.
Negative index values were replaced by 0 before obtaining these summaries.

Model selection and estimation of the accuracy of the chosen model were carried out using a rigorous procedure by splitting the data at random into training (50%), validation (25%), and test (25%) sets. This avoids the overly optimistic estimates of area under the curve (AUC), sensitivity, and specificity often obtained when the same data are used for model selection as well as for final estimation of prediction accuracy (Hastie *et al.*, 2001). Model parameters were estimated from the training set, and the corresponding AUC was estimated from the validation set.

Results from the model selection procedure, using only the training ($n = 239$) and validation ($n = 118$) sets, are summarized in Table 2. The AUC was used as the criterion for model selection as it is a summary of the whole receiver-operating characteristic curve for a given model and is not affected by choice of a cut point as is the case for sensitivity and specificity. The model with only $x4 = \log(1 + \text{IgG4})$ has an estimated AUC of 0.961 (from the validation set). Using additional predictors has a negligible effect on the AUC. Thus, the model with only IgG4 was chosen as the final model because of its parsimony and high AUC value. The classification rule thus developed is to declare IgG4 index values above 6.43 as cases, and values of 6.43 and below as non-cases.

Final estimates of AUC, sensitivity and specificity of the IgG4 classifier were obtained from the test set ($n = 118$). The estimated AUC of IgG4 is 0.97 (95% confidence interval (95% CI): 0.94–1.00), sensitivity is 92% (95% CI: 82–98%), and specificity is 97% (95% CI: 89–100%). The IgG4 classifier was developed entirely in the training and validation sets, yet it performed extremely well in the test set. The AUC for a test is the probability that a random participant from the disease group (patients) has a higher value than a randomly selected participant from the disease-free group (healthy controls). Our results indicate that with 95% CI, the AUC for IgG4 is higher than 0.94. This constitutes strong evidence that IgG4 is a reliable predictor.

Figure 1 shows smooth density plots of IgG and IgG subclass anti-Dsg1 index values (FS sera (red line) and

Table 2. Prediction models applied to the training set* (AUC, sensitivity, and specificity derived from validation set)**

Predictors	AUC	Sensitivity	Specificity
IgG1	0.78	0.74	0.78
IgG2	0.51	0.45	0.54
IgG3	0.81	0.81	0.68
IgG4	0.96	0.89	0.94
IgG	0.92	0.81	1.00
IgG1 and IgG2	0.80	0.72	0.78
IgG1 and IgG3	0.84	0.72	0.78
IgG1 and IgG4	0.97	0.91	0.95
IgG1 and IgG	0.91	0.81	1.00
IgG2 and IgG3	0.81	0.85	0.68
IgG2 and IgG4	0.96	0.91	0.95
IgG2 and IgG	0.94	0.83	1.00
IgG3 and IgG4	0.96	0.89	0.92
IgG3 and IgG	0.95	0.81	1.00
IgG4 and IgG	1.00	0.83	1.00
IgG1, IgG2, and IgG3	0.84	0.72	0.78
IgG1, IgG2, and IgG4	0.96	0.92	0.94
IgG1, IgG2, and IgG	0.94	0.83	1.00
IgG1, IgG3, and IgG4	0.97	0.91	0.94
IgG1, IgG3, and IgG	0.94	0.81	1.00
IgG1, IgG4, and IgG	1.00	0.92	0.94
IgG2, IgG3, and IgG4	0.97	0.83	1.00
IgG2, IgG3, and IgG	0.95	0.83	1.00
IgG2, IgG4, and IgG	1.00	0.87	1.00
IgG3, IgG4, and IgG	1.00	0.85	1.00
IgG1, IgG2, IgG3, and IgG4	0.97	0.92	0.95
IgG1, IgG2, IgG3, and IgG	0.95	0.83	1.00
IgG2, IgG3, IgG4, and IgG	1.00	0.87	1.00
IgG1, IgG2, IgG4, and IgG	1.00	0.83	1.00
IgG1, IgG3, IgG4, and IgG	1.00	0.87	1.00
IgG1, IgG2, IgG3, IgG4, and IgG	1.00	0.87	0.98

AUC, area under the curve.
*Each model was estimated from the training set (n=239).
**Parameter estimates were then applied to the validation set (n=118) to estimate the model's AUC, sensitivity, and specificity.

healthy donor sera (blue line)). The cut point of 6.43 chosen for IgG4 corresponds to a value of 2 on the x axis. It is clear that IgG4 anti-Dsg1 autoantibody index values produce the best separation between control and FS sera.

Positive and negative predictive values of the IgG4-based predictor

The accuracy of predictions generated by a given classifier in a population depends not only on its sensitivity and specificity, but also on the prevalence of disease in that population (Medina, 1999). Two important measures in that regard are the PPV and the NPV. The PPV is the probability that a participant classified as a case does indeed have the disease. The NPV is the probability that a participant classified as a non-case is actually disease-free**.** Table 3 shows how PPV and NPV depend on prevalence for a diagnostic test with sensitivity 92% and specificity 97%, which are the estimates for the IgG4-based classifier developed above. In a population such as that of Limao Verde with 3% prevalence, a randomly drawn participant has a 3% chance, or prior probability, of having FS. However, if that participant tests positive, the disease probability (PPV) goes up to nearly 49%. Similarly, a random participant from Limao Verde has a 97% of being disease-free. If that participant tests negative, the probability (NPV) of being disease-free goes up to 99.7%.

To compare IgG4 with other potential markers, sensitivity and specificity for IgG, IgG1, and IgG1 + IgG4 were estimated from the test set (n = 118). These sensitivity and specificity estimates differ from those in Table 2, which were obtained from the validation set. Table 3 shows the corresponding PPV and NPV for IgG1 (sensitivity: 72%, specificity: 80%), IgG (sensitivity: 87%, specificity: 91%), and the combination of IgG1 + IgG4 (sensitivity: 89%, specificity: 94%) for the population of Limao Verde. The PPVs for IgG1 was 10%, for IgG was 23%, and for the combination of IgG1 + IgG4 was 31% are lower than those of IgG4 anti-Dsg1.

Assessing the performance of the IgG4-based predictor

The performance of the classifier was evaluated in three additional sets of individuals:

(a) *FS cases from Limao Verde during the preclinical and clinical phases of the disease*. We have collected 11 FS cases for whom sera were available before they developed frank clinical FS. Some donors had several preclinical samples. The IgG4-based classifier found that 5 of the 11 cases (45%) had serological features of FS before developing clinical disease (cases 1, 3, 6, 9, and 11 of Table 4). The duration of the preclinical follow-up in each of the 11 cases is also presented in Table 4. The transition from preclinical to disease stage lasted 1 year (one case), 2 years (three cases), 3 years (one case), 4 years (one case), 7 years (three cases), and 10 years (two cases). The total IgG anti-Dsg1 index values are also included in Table 4. In 8 of the 11 cases the IgG anti-Dsg1 index values were positive. The five IgG4-positive cases were also positive for total IgG anti-Dsg1. The sera of three cases (nos. 7, 8, and 10) were positive for IgG anti-Dsg1 but negative for IgG4, indicating the different sensitivities and specificities of the total IgG and IgG4 anti-Dsg1 assays.

The 11 cases that underwent the transition of preclinical to clinical FS were further analyzed by comparing their serological features (IgG and IgG4 anti-Dsg1 autoantibodies) with a group of age-matched and sex-matched normal individuals from Limao Verde, known to have IgG anti-Dsg1 autoantibodies for the past 3–5 years and normal skin. The IgG4 classifier was positive in four of these individuals; three of them are relatives of one FS patient and maybe

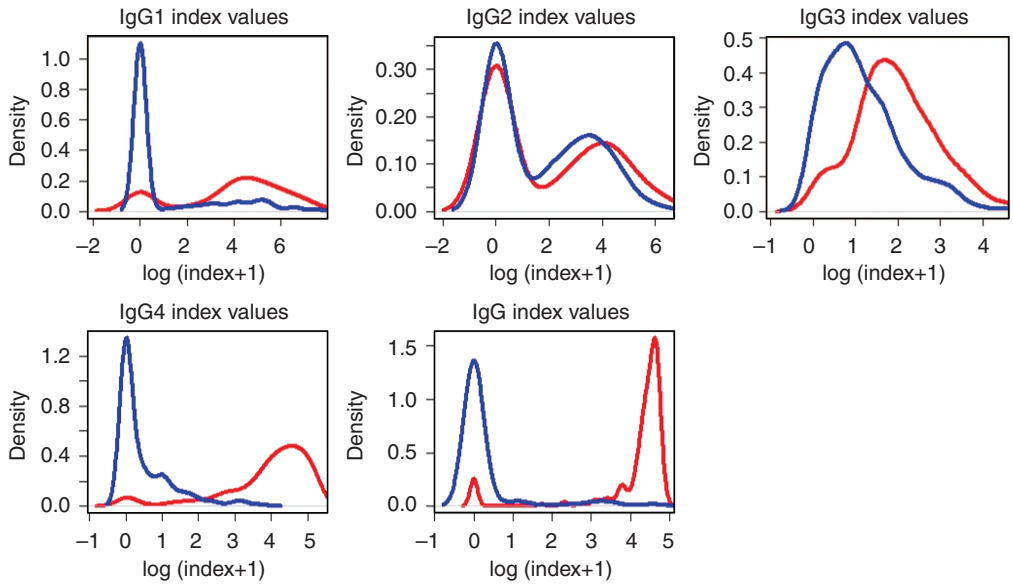

Figure 1. Smooth density plots of index values of anti-Dsg1 IgG subclasses and IgG in FS patients (red lines) and healthy donors (blue lines). Negative index values were converted to zero before applying the log transformation.

Table 3. PPV and NPV for IgG4, IgG1, IgG and IgG1+IgG4 anti-Dsg1 tests with different sensitivities and specificities applied to populations with various prevalence of disease

	IgG (Sens=87%, Spec=91%)		IgG1 (Sens=72%, Spec=80%)		IgG4 (Sens 92%, Spec=97%)		IgG1+IgG4 (Sens=89%, Spec=94%)	
Prevalence (%)	**PPV**	**NPV**	**PPV**	**NPV**	**PPV**	**NPV**	**PPV**	**NPV**
0.1	0.00958	0.99986	0.00359	0.99965	0.02978	0.99992	0.01463	0.99988
1	0.08896	0.99856	0.03509	0.99648	0.23650	0.99917	0.13031	0.99882
3[1]	0.23016	0.99560	0.10019	0.98929	0.48677	0.99746	0.31449	0.99639
10	0.51786	0.98438	0.28571	0.96257	0.77311	0.99092	0.62238	0.98716
20	0.70732	0.96552	0.47368	0.91954	0.88462	0.97980	0.78761	0.97158
80	0.97479	0.63636	0.93506	0.41667	0.99191	0.75194	0.98343	0.68116
90	0.98864	0.43750	0.97006	0.24096	0.99639	0.57396	0.99257	0.48705
99	0.99896	0.06604	0.99720	0.02805	0.99967	0.10911	0.99932	0.07946
99.9	0.99990	0.00696	0.99972	0.00285	0.99997	0.01199	0.99993	0.00848

The specificty and sensitivity derived from the test set (25% of the total data set) as described in Methods and Results.
PPV, positive predictive value; NPV, negative predictive value
[1]The Amerindian reservation of Limao Verde, Brazil exhibits a prevalence of Fogo Selvagem of ~3%.

genetically predisposed. We are following these individuals closely for evidence of FS.

(b) *Sera from Japanese patients with PV and PF.* Sera from 20 mucosal PV patients, which were known to contain only anti-Dsg3 autoantibodies, were classified as normal sera by the IgG4 anti-Dsg1 classifier. In contrast, the sera of 17 of 20 mucocutaneous PV (mcPV) and 18 of 20 PF were classified as having features of FS as they possess anti-Dsg1 autoantibodies. The sera of Japanese PF patients contain anti-Dsg1 autoantibodies, predominantly IgG4 (data not shown). Moreover, the sera of mcPV patients contain a combination of anti-Dsg1 and anti-Dsg3 autoantibodies.

(c) *Sera from three Limao Verde cohorts (2005 data).* The IgG4-based classifier was applied to the sera of 96 individuals, members of the three cohorts from the Limao Verde Reservation under study since 2005, when the initial evaluation was performed and serum samples were obtained. The IgG4 classifier identified 21 individuals (22%) with serological features of FS: 6 of 34 individuals (17.6%) in cohort 1 were positive, 6 of 39 (15.3%) in cohort 2 were also positive as well as 9 of 24 (37.5%) in cohort 3 (Table 5). In addition, the same donors show the following percentages of total IgG anti-Dsg1 autoantibodies: cohort 1 (1/34; 2.9%), cohort 2 (3/41; 7.3%), and cohort 3 (7/24; 29%) (Diaz *et al.*,

Table 4. IgG and IgG4 anti-Dsg1 autoantibodies in 11 FS cases during the preclinical and clinical stage of the disease

		1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2004	2005	Preclinical (years)
Case 1	IgG	94.34	Unavailable	69.92										2
FS-27	IgG4	95.73	Unavailable	87.66										
Case 2	IgG	50.08	Unavailable	Unavailable	70.76									3
FS-29	IgG4	1.49	Unavailable	Unavailable	31.5									
Case 3	IgG			56.63	Unavailable	27.38								2
FS-31	IgG4			9.2	Unavailable	17.75								
Case 4	IgG			108.03	Unavailable	104.18								2
FS-32	IgG4			3.2	Unavailable	24.79								
Case 5	IgG	30.02	Unavailable	Unavailable	Unavailable	50.76								4
FS-33	IgG4	1.29	Unavailable	Unavailable	Unavailable	59.12								
Case 6	IgG	68.99	121.01	Unavailable	Unavailable	27.83	Unavailable	ND	Unavailable	45.07	−45.85	4.17		10
FS-45	IgG4	6.87	7.04	Unavailable	Unavailable	3.99	Unavailable	0.65	Unavailable	26.36	16.74	18.84		
Case 7	IgG		−8.88	Unavailable	Unavailable	−19.98	Unavailable	−25.87	Unavailable	ND	49.9	−31.10	103.42	10
FS-46	IgG4		1.33	Unavailable	Unavailable	0.22	Unavailable	12.56	Unavailable	0.17	0.7	32.83	28.76	
Case 8	IgG	−17.66	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	37.52					7
FS-37	IgG4	1.38	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	12.42					
Case 9	IgG	73.06	Unavailable	Unavailable	Unavailable	84.31	Unavailable	Unavailable	136.63					7
FS-38	IgG4	33.29	Unavailable	Unavailable	Unavailable	96.02	Unavailable	Unavailable	137.83					
Case 10	IgG	−88.01	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	27.21					7
FS-39	IgG4	−0.46	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	Unavailable	11.26					
Case 11	IgG		71.89	169.5										1
BA1-1	IgG4		16.09	82.48										

Positive IgG4 index value: >6.43.
Positive IgG index value: >11.34.
FS, fogo selvagem; ND, not determined.

2008). From the 21 cohort members showing positive IgG4 anti-Dsg1 autoantibodies, 12 had IgG4 autoantibodies alone (57%) and 9 had a combination of IgG and IgG4 autoantibodies (42.8%). Only 2 individuals outside the 21 were positive for IgG anti-Dsg1 alone. The remaining 75 healthy cohort members exhibited negative IgG anti-Dsg1 autoantibodies. These results demonstrate the higher sensitivity of the IgG4 classifier to detect anti-Dsg1 autoantibodies. It is interesting that a member of cohort three classified as FS by our IgG4 predictor (individual JDM) has developed clinical FS in 2005.

DISCUSSION

The development of an autoimmune disease is one of the fundamental enigmas of immunology. Many genetic and environmental factors appear to play a role, making it difficult to fulfill Koch's criteria for any of these diseases. Some of the advantages that an organ-specific autoimmune disease such as FS (which is epidermal-specific) offers to research in autoimmunity are related to the fact that anti-Dsg1 autoantibodies are pathogenic and the self-antigen, Dsg1, is fully characterized. Dissection of the underlying mechanisms involved in autoantibody formation is a difficult task because

Table 5. Individuals from Limao Verde, Brazil, identified as FS cases by the IgG4 predictor

	Age (years)	IgG4 index values
Cohort 1 (ages 5–10)		
C1-1	5.4	7.32
C1-2	5.6	11.17
C1-3	6.3	6.43
C1-4	7.7	7.09
C1-5	8.2	15.17
C1-6	9.7	8.19
Cohort 2 (ages 11–15)		
C2-1	10.7	130.62
C2-2	11.1	34.88
C2-3	11.5	40.87
C2-4	11.8	12.36
C2-5	12.4	31.12
C2-6	14.2	57.55
Cohort 3 (ages 16–20)		
C3-1	15.1	47.15
C3-2	15.5	25.63
C3-3	15.5	19.89
C3-4	16.8	34.09
C3-5	17.3	121.73
C3-6	17.9	21.29
C3-7[1]	18.4	56.75
C3-8	19.8	52.39
C3-9	20.3	34.68

FS, fogo selvagem.
[1]Developed FS during the course of study.

in many instances it is not possible to differentiate between the pathogenic autoantibody response and a normal background (natural) immune response.

Importantly, IgG autoantibodies against Dsg1 are detected not only in FS patients but also in a significant number of healthy individuals living in an endemic region such as Limao Verde (Hans-Filho *et al.*, 1996; Diaz *et al.*, 2008). In this settlement, we have witnessed the transition from preclinical to clinical stage of the disease (Warren *et al.*, 2000, 2003; Li *et al.*, 2003; Diaz *et al.*, 2008). The challenge therefore is to develop assays to detect the earliest serological markers of FS during the preclinical stage. As shown in this study, we have developed a classifier/predictor by analyzing the IgG and the four IgG subclass anti-Dsg1 autoantibody response in a large data set derived from FS patients and healthy controls (Table 1) using a sensitive and specific Dsg1 ELISA for IgG and each IgG subclass.

A set of 475 serum samples were tested for IgG and IgG subclass anti-Dsg1 and were divided at random into a training set ($N = 239$), a validation set ($N = 118$), and a test set ($N = 118$) to select a prediction model and to assure unbiased estimation of classification accuracy. The best classifier involves only IgG4 with an estimated AUC of 0.97 (95% CI: 0.94–1.00), sensitivity of 92% (95% CI: 82–98%), and specificity of 97% (95% CI: 89–100%) (Table 3 and Figure 1). The sensitivity and specificity for the IgG assay for anti-Dsg1 autoantibodies were 5% and 6% lower than those of IgG4 predictor. The PPV and NPV of the classifier, when applied in a population such as the Limao Verde Reservation with 3% prevalence, were calculated. It is estimated that an individual classified as positive has a 49% chance of having FS whereas a participant classified as negative has a 99.7% probability of being disease-free (Table 3). The PPVs for IgG1 (10%), IgG (23%), and IgG1 + IgG4 (31%) anti-Dsg1 autoantibodies were lower than IgG4. Although the IgG anti-Dsg1 test also performed well as a serological marker of FS (Table 2 and Figure 1), the AUC of IgG (0.92) was lower than the IgG4 AUC (0.96). In addition, using the sensitivity and specificity for the IgG and IgG4 assays, and the 3% prevalence of FS in Limao Verde, we estimated that 8.5% of the inhabitants of this reservation would have a false-positive IgG anti-Dsg1 assay. The false-positive tests using the IgG4 anti-Dsg1 assay, however, would be only 2.9%. Finally, since normal inhabitants of endemic areas of FS, such as Limao Verde, possess anti-Dsg1 autoantibodies (Warren *et al.*, 2000; Diaz *et al.*, 2008), its use as a classifier in these human settlements would be very limited. In contrast, IgG4 anti-Dsg1 autoantibodies are detected in the sera of individuals developing FS or during recurrence (Li *et al.*, 2003; Warren *et al.*, 2003). For similar reasons the IgM anti-Dsg1 autoantibodies (Diaz *et al.*, 2008) did not perform well (data not shown).

The IgG4-based classifier was validated further by analyzing two groups of patients: a group of 11 FS patients during the preclinical stage of the disease and a group of 60 Japanese PV and PF patients. In the first group, the classifier predicted FS in 5 of 11 individuals (45%) during the preclinical stage and in all samples during the clinical stage of FS (100%). It must be emphasized that this classifier identifies participants with serological features of FS regardless of the presence of active skin disease. We propose that this IgG4-based classifier is a serological marker of FS during the preclinical and clinical stages of the disease. During the preclinical stage, this classifier may show variations over time because of fluctuations in environmental antigenic stimulation as observed in cases 6 and 7 in Table 4. However, once a participant exhibits skin disease, the classifier will be positive with high probability, provided that the serum sample is obtained before the initiation of systemic therapy. Eight of the eleven patients exhibited IgG anti-Dsg1 autoantibodies during the preclinical stage of FS, in agreement with the high prevalence of these IgG autoantibodies in this human settlement. Similarly, a control group from Limao Verde composed of normal individuals with positive IgG anti-Dsg1 autoantibodies, matched by age and sex with the 11 patients in Table 4, includes 4 normal participants with positive IgG4 anti-Dsg1. Three of these normal individuals were first-degree relatives of a patient with FS. These individuals have been under close

observation for 3–5 years since the collection of serum samples. We will continue following them for any evidence of clinical FS.

In the group of 60 Japanese patients, 20 patients with mucosal PV, possessing only anti-Dsg3 autoantibodies, were classified as normal donors (because of the absence of anti-Dsg1 autoantibodies). The IgG4-based classifier performed well in a group of 20 PF patients, of whom 18 were identified as cases. In a group of 20 mcPV patients, possessing anti-Dsg1 and anti-Dsg3 autoantibodies, the classifier predicted the disease in 17 cases. Hence, the IgG4-based classifier performed well in the Japanese group of patients with PF and mcPV as both groups of patients possess anti-Dsg1 autoantibodies.

The IgG4-based classifier was also used to test the members of an ongoing prospective cohort in the Limao Verde Reservation including 96 donors, aged 5–20 years on their first evaluation in 2005. The IgG4 classifier identified 21 individuals (22%) with serological features of FS: 6 of 34 individuals (17.6%) in cohort 1 were positive, 6 of 39 (15.3%) in cohort 2 were also positive as well as 9 of 24 (37.5%) in cohort 3 (Table 5). The same donors show the following percentages of total IgG anti-Dsg1 autoantibodies: cohort 1 (1/34; 2.9%), cohort 2 (3/41; 7.3%), and cohort 3 (7/24; 29%) (Diaz *et al.*, 2008). Interestingly, one member of the third cohort (JDM), classified as a case, has developed FS in the course of the study. According to the PPV of the IgG4 classifier, it is estimated that about 50% of these positive participants from Limao Verde have FS in the preclinical stage and are at risk to develop clinical disease if the conditions are appropriate. Similarly, each of the 75 participants identified as normal by the classifier have a 99% chance of being disease-free. Forecasting active clinical disease in individuals of both groups (positive and negative) using the classifier is the subject of current investigation in our laboratory. An ongoing prospective study of these cohorts will validate further this immunological instrument not only as an identifier of current FS serology but also as a predictor of future disease. These cohorts are evaluated clinically every 4 months and serologically every 2 years.

Organ-specific and non-organ-specific autoantibodies have been reported as diagnostic aids in diseases such as type I diabetes, thyroiditis, adrenalitis, myasthenia gravis, systemic lupus erythematosus, and rheumatoid arthritis (Leslie *et al.*, 2001; Scofield, 2004). Moreover, in some of these diseases the respective autoantibodies have been detected years before the onset of clinical disease (Notkins and Lernmark, 2001; Arbuckle *et al.*, 2003). The value of autoantibodies as predictors has been reviewed (Arbuckle *et al.*, 2003; Scofield, 2004) and is well documented in diabetes (Notkins and Lernmark, 2001). In diabetes, the presence of autoantibodies against glutamic acid decarboxylase, protein tyrosine phosphatase-like molecule, and insulin in healthy individuals could predict the development of disease in more than 70% of first-degree relatives of the cases in the course of 2–10 years (Notkins and Lernmark, 2001). This report moves FS into the group of organ-specific autoimmune diseases where epidermal-specific autoantibodies can be used not only as diagnostic markers of FS but also as predictors of disease as shown in 6 of 11 FS cases where preclinical sera were available.

Finally, we are reporting an IgG4-based classifier that is able to identify a serum as exhibiting features of FS or normal serum. After the ELISA anti-Dsg1 assay methodology to test for IgG subclasses reported in this study, we found that IgG4 index values above 6.43 are sufficient to classify a serum sample as having features of FS. Its usefulness in the evaluation of PF patients or in endemic forms of PF in other regions of the world must be validated as FS and the endemic regions of FS are unique. However, our study suggests that the IgG4-based classifier can be extremely useful in identifying individuals during the preclinical stage of FS. HLA typing and the IgG4-based classifier would become powerful tools for the selection of individuals to undergo close clinical and serological surveillance. Moreover, as the environmental risk factor(s) can also be assessed among potential FS patients, these immunological markers may enhance our ability to identify these factor(s) involved in triggering the autoimmune disease in FS.

MATERIALS AND METHODS

Sources of sera

A total of 475 serum samples were tested for IgG subclass anti-Dsg1 in this investigation, 214 from FS cases and 261 from healthy controls (Table 1). From this set 459 sera were tested for IgG anti-Dsg1 autoantibodies (209 FS and 250 controls) (Table 1). Sera from classic cases of FS were collected from six Brazilian hospitals dedicated to treat these patients: Hospital das Clinicas, Sao Paulo (Hospital SP), ($n=49$); Hospital de Doenças Tropicaes, Goiania (Hospital GO), ($n=41$); Hospital Adventista do Penfigo, Campo Grande (Hospital CG) ($n=27$), Hospital Universitario de Belho Horizonte, Minas Gerais (Hospital MG) ($n=47$), Hospital Universitario de Brasilia (Hospital Br ($n=47$), and Outpatient Clinic, Cascavel, Parana (Parana ($n=3$). The FS sera were derived from patients with widespread skin disease. Clinically, they show superficial blisters and erosions and histologically subcorneal vesicles. The indirect immunofluorescence studies showed anti-epidermal ICS autoantibodies in titers above 1:80. FS patients admitted to Brazilian hospitals with widespread disease comprised individuals with the generalized forms of the disease as described earlier (Hans-Filho *et al.*, 1999). They include the bullous exfoliative, the exfoliative erythrodermic, and forms characterized by generalized keratotic plaques and nodular lesions. The study was conducted according to the Declaration of Helsinki Principles. Participants gave their written informed consent.

Normal human sera were obtained from blood bank donors from Hospital SP ($n=57$), BH ($n=32$), Hospital GO ($n=41$), and the University of North Carolina Blood Bank ($n=131$).

Sera used to validate the IgG4 predictor

(a) Eleven sera from FS patients before and after the onset of FS.
(b) Sera from Japanese patients with PF and PV (Professor M. Amagai from Keio University, Tokyo, kindly provided us with sera from the following groups of patients: Dsg1-positive PF

($n=20$), Dsg3-positive mucosal PV ($n=20$), and Dsg1-positive, Dsg3-positive mcPV ($n=20$)).

(c) Sera from three Limao Verde cohorts.

Currently, we are following three cohorts, all inhabitants of the Limao Verde Reservation. The first cohort comprises normal donors of age 5–10 years ($n=34$); the second, age 11–15 years ($n=38$); and the third, age 16–20 years ($n=24$). The sera were obtained during the first evaluation of the cohorts in May 2005. These investigations were approved by the Institutional Review Boards of the University of North Carolina and the University of Sao Paulo, Brazil.

Production and purification of recombinant Dsg1

A His-tagged recombinant form of Dsg1, encompassing the extracellular domain of this protein, was generated in the baculovirus system and purified by nickel affinity chromatography using the procedure of Ding *et al.* (1997). Optimum conditions for this expression system were determined empirically as described by Liebman *et al.* (1999). The typical protein yield was 10 μg/ml of culture supernatant.

IgG and IgG subclass anti-Dsg1 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 (TBS/Ca^{2+}/T-20), the plate was blocked with 1% BSA in TBS/Ca^{2+}/T-20 at room temperature for 1 hour. The plate was then incubated with duplicate 1:100 dilution (for IgG plates) and 1:50 dilution (for IgG subclasses) of serum samples for 1 hour at room temperature. After washing, the plate was incubated with a 1:2,000 dilution (for IgG plates) and 1:1,000 dilution (for IgG subclasses) of a mouse horseradish peroxidase-labeled mAb against human IgG or human IgG1, IgG2, IgG3, and IgG4 subclasses (Zymed, San Francisco, CA) (Warren *et al.*, 2003; Diaz *et al.*, 2008). 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 optical density (OD) as follows:

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

Statistical analysis

A logistic regression model (McCullagh and Nelder, 1989) was used to develop a classifier that predicts case–control status based on IgG and the four IgG subclass index values. The predictors were defined as follows: first, negative IgG index values were replaced by 0, then predictors were computed as $x = \log(\text{IgG} + 1)$. This removed much of the skewness in the IgG index values. Thus, x_1 through x_5 were derived from IgG1 through IgG4 and total IgG. For the purpose of developing and evaluating a classification rule, the data set was divided at random into three parts: a training set, a validation set, and a test set, containing 50, 25, and 25%, respectively, of cases and controls. This follows the guidelines given by Hastie *et al.* (2001).

The following procedure was applied to choose the best model for prediction. All 31 possible models, containing an intercept and from 1 to 5 predictors, were considered. Each model was estimated from the training set. Parameter estimates were then applied to the validation set to estimate the model's AUC. For example, the model ''IgG1, IgG2, and IgG3'' in Table 2 is a logistic regression model with linear predictor $\beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3$. Fitting the model to the training set yields estimates b_0, b_1, b_2, and b_3. A score was then computed for each participant in the validation set as score $= b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3$. The score was used to compute the AUC as a measure of the model's predictive ability. AUC was estimated using non-parametric methods (Hanley and McNeil, 1982).

Additionally, the score was transformed to the probability scale by $P = 1/\{1 + \exp(-\text{score})\}$. For the purpose of computing sensitivity and specificity, if $P > 0.45$ (equivalent to score above -0.2), the participant was classified as a case, otherwise as a non-case. Cut points other than 0.45 were evaluated, but 0.45 was deemed to provide the best tradeoff between sensitivity and specificity. Sensitivity and specificity were estimated as binomial proportions. The analysis was carried out using SAS 9.1 and R 2.5 software.

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

This project was supported by NIH Grants R01-AR30281, RO1-AR32599, and T32 AR07369 (L.A.D.).

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