Antigen Selection of Anti-DSG1 Autoantibodies During and Before the Onset of Endemic Pemphigus Foliaceus

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Fogo selvagem (FS), the endemic form of pemphigus foliaceus (PF), is characterized by pathogenic anti-desmoglein 1 (DSG1) autoantibodies. To study the etiology of FS, hybridomas that secrete either IgM or IgG (predominantly IgG1 subclass) autoantibodies were generated from the B cells of eight FS patients and one individual 4 years before FS onset, and the H and L chain V genes of anti-DSG1 autoantibodies were analyzed. Multiple lines of evidence suggest that these anti-DSG1 autoantibodies are antigen selected. First, clonally related sets of anti-DSG1 hybridomas characterize the response in individual FS patients. Second, H and L chain V gene use seems to be biased, particularly among IgG hybridomas, and third, most hybridomas are mutants and exhibit a bias in favor of CDR (complementary determining region) amino acid replacement (R) mutations. Strikingly, pre-FS hybridomas also exhibit evidence of antigen selection, including an overlap in V_H gene use and shared multiple R mutations with anti-DSG1 FS hybridomas, suggesting selection by the same or a similar antigen. We conclude that the anti-DSG1 response in FS is antigen driven and that selection for mutant anti-DSG1 B cells begins well before the onset of disease.

Journal of Investigative Dermatology (2009) **129,** 2823–2834; doi:10.1038/jid.2009.184; published online 2 July 2009

INTRODUCTION

Pemphigus encompasses a group of autoimmune blistering diseases exhibiting pathogenic autoantibodies against desmogleins (DSG), a family of desmosomal cell adhesion glycoproteins (Lever, 1953; Beutner and Jordon, 1964; Ding *et al.*, 1997; Udey and Stanley, 1999). The hallmark of these diseases is the presence of intraepidermal vesicles (Lever, 1953) and anti-epidermal autoantibodies (Beutner and Jordon, 1964; Ding *et al.*, 1997; Udey and Stanley, 1999). Pemphigus foliaceus (PF) and pemphigus vulgaris (PV) are the two major phenotypes of pemphigus (Lever, 1953). Immunologically, the sera of PF patients show anti-DSG1 antibodies, whereas the sera of PV patients contain antibodies against DSG3 (mPV), or both DSG1 and DSG3 (mcPV) (Ding *et al.*, 1997; Udey and Stanley, 1999). PV and PF in North America are sporadic (Lever, 1953; Udey and Stanley, 1999), but endemic PF is described in certain states of Brazil, where it is known as fogo selvagem (FS) (Diaz *et al.*, 1989b). FS shows similar clinical, histological, and immunological features to those observed in non-endemic PF (Stanley *et al.*, 1986; Diaz *et al.*, 1989a). The published epidemiological studies of FS strongly suggest that this disease is precipitated by environmental agent(s) harbored in certain regions of Brazil. One of these sites, under investigation by our group for the last 15 years, is the Amerindian Reservation of Limao Verde (Hans-Filho *et al.*, 1996). We have reported the serological transition from preclinical to clinical stage of FS in several cases from Limao Verde (Warren *et al.*, 2000, 2003; Li *et al.*, 2003; Qaqish *et al.*, 2009).

Fogo selvagem is mediated by pathogenic autoantibodies against DSG1 (Roscoe *et al.*, 1985; Stanley *et al.*, 1986). These pathogenic autoantibodies are IgG4 restricted (Rock *et al.*, 1989) and their appearance in the serum heralds the onset of clinical disease (Warren *et al.*, 2003). In fact, a recent study by our group has identified IgG4 anti-DSG1 autoantibodies as the serological markers of disease in FS (Qaqish *et al.*, 2009). Non-pathogenic IgG anti-DSG1 autoantibodies (Warren *et al.*, 2000; Li *et al.*, 2003) and IgM anti-DSG1 autoantibodies (Diaz *et al.*, 2008) have been detected in healthy individuals living in endemic areas of FS. The underlying mechanism of autoantibody formation in FS, however,

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Abbreviations: CDR, complementary determining region; DSG1, desmoglein 1; FS, fogo selvagem; FWR, framework region; PBMC, peripheral blood mononuclear cell; PF, pemphigus foliaceus; PV, pemphigus vulgaris; R, replacement mutations; S, silent mutations

Received 4 February 2009; revised 23 April 2009; accepted 15 May 2009; published online 2 July 2009

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specifically that of anti-DSG1 autoantibodies, is still poorly understood. Whether these autoantibodies are developed through a polyclonal activation or an antigen-driven mechanism is still a mystery, but this information is needed to identify the cause of FS. To provide a definite answer to this question requires the genetic analysis of anti-DSG1 autoantibody gene repertoire.

Analyses of the V gene sequences encoding the autoantibodies in PV by our group (Qian *et al.*, 2007) and by Payne *et al.* (2005) have yielded important clues to the development of anti-DSG1 and anti-DSG3 antibodies. The potentially pathogenic IgG anti-DSG response in PV has been shown to be antigen selected (Qian *et al.*, 2007). The results of these studies are similar to the findings reported in other autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis. Recently, two single-chain variable fragments of pathogenic autoantibodies from a PF patient were isolated and the H chain V regions of the autoantibodies from this patient were shown to be encoded by a restricted number of genes (Ishii *et al.*, 2008).

Although there has been no genetic study on the autoantibodies in individuals before the onset of autoimmune diseases, the high prevalence of FS in Limao Verde provides us with a unique opportunity to address this question, as we have preserved peripheral blood mononuclear cells (PBMCs) of selected FS and healthy individuals from this human settlement. In this study, we have examined anti-DSG B cells from multiple FS patients and from an individual who was healthy at the time of the blood draw, but developed FS 4 years later. We conclude that (a) anti-DSG response in FS is composed of mutant B cells that have been subject to extensive antigen selection, and (b) the pre-clinical anti-DSG response is composed of mutant B cells that have undergone the same or similar selection pressures. These findings indicate the presence of inciting antigen(s) in FS endemic areas, and these inciting antigen(s) have an important role in the etiology of FS.

RESULTS

Anti-DSG1 in FS patients is oligoclonal

We fused Epstein-Barr virus-transformed PBMCs from eight FS patients with myeloma cells and screened for the resulting hybridomas for anti-DSG1 production by ELISA. We generated 78 anti-DSG1 hybridomas, 40 IgM producers, and 38 IgG producers (Table 1). Most IgG anti-DSG1 hybridomas were IgG1. Despite the fact that the IgG4 autoantibodies are the main pathogenic antibodies in the sera of FS patients (Rock *et al.*, 1989), only two IgG4 hybridomas were identified from two FS patients. There are possible explanations for the disparity between the high levels of serum IgG4 from FS patients and the low frequency of IgG4 hybridomas generated in this study. First, serum IgG4 antibodies were produced by plasma cells. Plasma cells reside mainly in the tissues and bone marrow, and are rarely present in peripheral blood (Benner *et al.*, 1981; Slifka and Ahmed, 1996). Thus, they were not abundantly present in the FS patients' blood samples collected. Second, Epstein-Barr virus transformation predominantly immortalizes B cells (Middleton *et al.*, 1991), and the IgG4-secreting plasma cells might not be immortalized and thus might be absent from the subsequent hybridoma generation. Third, this may simply reflect the relative amount of IgG1- and IgG4-expressing B cells in the peripheral blood of FS patients. The DSG1 specificity of the antibodies produced by these hybridomas was confirmed by immunoprecipitation (Figure 1a) and indirect immunofluorescence (Figure 1b). Most of these hybridomas (35 of 38) also recognized the ectodomain of DSG3 by ELISA (data not shown).

The mRNA of the expressed V_H and V_L genes of these 77 anti-DSG1 hybridomas was PCR amplified and sequenced to determine V_H and V_L gene use and to identify somatic mutations. Table 1 summarizes this analysis. Clonally related sets of hybridomas from an individual fusion are a hallmark of secondary responses to foreign antigen and are also a characteristic of autoimmune responses, including those in human PV, as we have shown previously (Qian *et al.*, 2007). This oligoclonality is because of the antigen-driven clonal expansion of a limited number of B-cell clones. Clonally related hybridomas express the identical V region genes and have identical V_H CDR3 sequences. By these criteria, we identified 12 sets of clonally related hybridomas from seven of the eight FS patients. In patient FS8, all seven of the anti-DSG1 hybridomas identified belonged to a single clonal set, whereas those from patients FS12 and FGS belonged to only two clonal sets each, indicating the dominance of a small number of clones in some patients. Most clonally related hybridomas were identical in sequence, suggesting that a limited number of clones in each patient had undergone extensive clonal growth in the absence of somatic mutation. However, sequence identity raises the possibility that the presence of clonal sets is an artifact of *in vitro* growth. To discriminate between these possibilities, we compared the extent of somatic mutation among the clonal sets before divergence with that of singlet hybridomas. On the basis of sequence comparison with the most similar germline V_H genes, most (9 of 11) clonally related sets had at least 10 differences from germline, indicating the occurrence of extensive somatic mutation before clonal divergence. This was significantly different from that of singlet hybridomas, in which only half had 10 or more mutations (16 of 35) (χ^2, $P=0.036$). This argues against an *in vitro* artifact to explain the presence of clonal sets in FS patients, as any *in vitro* expansion will be independent of the extent of somatic mutation. This is further suggested by intraclonal sequence difference in the clonal set from patient FS12 (FS12-1F10 and FS12-3A7). Thus, the presence of clonal sets of hybridomas in these FS patients likely reflects *in vivo* clonal expansion, presumably because of the selective advantage in antigen binding conferred by the somatic mutations they acquired before clonal divergence.

We identified 18 V_H genes used by 48 clonally independent anti-DSG1 hybridomas (Table 1 and Figure 2a upper panels) and found that IgM and IgG anti-DSG1 hybridomas differed significantly in their expressed V_H repertoires. The V_H3 gene family use increased from 43.5% among IgM hybridomas to 68.2% (χ^2, $P=0.095$) among IgG hybridomas,

Table 1. Anti-desmoglein 1 (Dsg1) hybridomas from fogo selvagem (FS) patients

					FWRs			CDRs				
PBMC	Clones[1]	Isotype	V_H	Similarity	R^2	S^3	P^4(FWRs)	R	S	P(CDRs)	J_H	CDR3
FS6	FS6-1D10	IgM	IGHV3-48*02	98.3	1	0	0.06073	4	0	**0.00262**	IGHJ4*02	DSGYNTFDY
	FS6-2C12	IgG1	IGHV4-59*01	90.0	9	8	**0.00311**	9	3	**0.04149**	IGHJ5*02	QNCSPNSCFNYFAP
	FS6-2F5(6)	IgM	IGHV3-23*01	94.2	8	7	0.19667	2	0	0.70918	IGHJ4*02	LIDGGY
	FS6-2G3	IgG1	IGHV3-23*01	92.2	5	5	**0.00031**	12	1	**0.00010**	IGHJ6*02	EMSLQPVAGMDV
	FS6-3A4(2)	IgG1	IGHV1-69*01	94.9	3	7	**0.00359**	3	1	0.33725	IGHJ6*03	GRFYHMDV
	FS6-3D7	IgM	IGHV1-18*01	95.9	4	2	0.05007	5	1	**0.03033**	IGHJ4*02	DLGGTYFDY
	FS6-4A3	IgM	IGHV4-4*07	100.0							IGHJ6*03	DRGVGYCSSTSCYSGRYMDV
	FS6-4D4	IgM	IGHV1-3*01	96.6	7	2	0.76103	0	1	0.93314	IGHJ4*02	HYYDSSGYYDNFDY
	FS6-5B7	IgM	IGHV2-5*05	98.3	3	2	0.53435			0.81007	IGHJ4*02	STGAARTDNTYYFDY
FS7	**FS7-1C11**	IgG1	IGHV5-51*01	97.0	3	2	**0.03761**	5	0	**0.01121**	IGHJ4*02	HRIGYCSGSNCYDFDY
	FS7-1H10	IgG2	IGHV1-18*01	91.3	6	9	**0.00038**	5	5	0.38273	IGHJ4*02	HFIPAPPDY
	FS7-2A9	IgG1	IGHV3-23*02	95.2	2	3	**0.00065**	8	1	**0.00054**	IGHJ4*02	RDIYGDVGVGLVDY
	FS7-2F8(4)	IgG1	IGHV3-7*01	91.5	8	6	**0.01155**	9	1	**0.01347**	IGHJ4*02	TESATIFGVAYYYFDY
	FS7-2G6	IgM	IGHV3-23*01	95.3	5	3	0.05574	3	3	0.33725	IGHJ3*02	DPGAPCSTTNCYVSDAFDM
	FS7-4C1	IgG1	IGHV3-23*01	96.6	3	1	**0.04677**	5	1	**0.01110**	IGHJ6*03	DSDSHYYMDV
	FS7-4H7	IgM	IGHV5-51*01	95.2	5	2	**0.04332**	5	2	0.05366	IGHJ4*02	GGEAYNLDY
FS8	*FS8-1A9(7)*	IgM	IGHV4-59*01	96.6	3	3	0.05258	2	2	0.40536	IGHJ6*03	GVYYGSGGYYGGGSYYYMDV
FS12	*FS12-1D2(2)*	IgG1	IGHV3-7*01	96.3	2	2	**0.00558**	3	4	0.23564	IGHJ4*02	DSLTAYCGGDCPTVTFGY
	FS12-1F10(2)	IgG1	IGHV3-74*01	99.0	0	1	**0.03950**	2	0	**0.04735**	IGHJ3*02	AYYDFWSGHDDAFDI
	FS12-3A7	IgG1	IGHV3-74*01	99.3	0	1	0.09205	1	0	0.18335	IGHJ3*02	AYYDFWSGHDDAFDI
FS33	FS33-1A9	IgM	IGHV1-46*01	100.0							IGHJ3*02	DRPDSSGYYLGAFDI
	FS33-1B8(4)	IgM	IGHV3-7*01	98.6	0	2	**0.01657**	2	0	0.09153	IGHJ6*04	DRQGIYYYYGLDV
	FS33-1D4(2)	IgM	ND									
	FS33-2B12	IgG1	IGHV3-48*03	91.8	9	9	**0.02832**	5	1	0.35950	IGHJ5*02	GLPYSGSDRGLDP
	FS33-2C6	IgG1	IGHV3-13*01	97.9	3	0	0.36241	3	0	**0.03787**	IGHJ4*02	RRVIRVRGVIPFFDY
	FS33-2E6(4)	IgG1	IGHV3-11*03	90.1	8	8	**0.00121**	9	3	**0.04319**	IGHJ4*02	GIDYYDSSGHYGSWGEDR
	FS33-2F4	IgG1	IGHV4-39*06	96.6	3	1	**0.03664**	7	0	**0.00080**	IGHJ4*02	DWGTGWQPLNYFDY
	FS33-3C6	IgM	IGHV1-3*01	98.3	2	1	0.22538	2	0	0.13601	IGHJ4*02	STRITMITSGY
	FS33-3C7	IgG1	ND									
	FS33-3E5	IgM	IGHV1-46*01	96.6	4	1	0.13579	3	2	0.18203	IGHJ4*02	DPGRGAAGIGYYFDY
	FS33-3H3	IgM	IGHV3-30*18	97.0	2	1	**0.02074**	4	2	**0.03590**	IGHJ6*02	ERTVATLYHYYYYGMDV
	FS33-4D4(2)	IgM	IGHV1-3*01	89.1	12	8	**0.01015**	8	4	0.16514	IGHJ5*02	NQQQLEQQNWYVP
	FS33-4G10	IgM	IGHV1-69*01	99.7			0.21008	0	1	0.58756	IGHJ4*02	SGYDFYSADY
	FS33-5E11	IgM	IGHV1-46*01	100.0							IGHJ3*02	DRPDSSGYYLGAFDI
	FS33-5F5	IgG1	IGHV5-51*01	99.0	0	1	**0.03400**	2	0	**0.04351**	IGHJ4*02	RTMATITGPIGY
	FS33-5F7	IgM	IGHV3-73*02	98.7	2	1	0.39825	0	1	0.77521	IGHJ5*02	GRDGTVVSLGST
GCDS	*GCDS-1C2(2)*	IgG1	IGHV3-23*01	95.6	4	4	**0.03017**	4	1	0.12097	IGHJ4*02	RRFDWLLYFDY
	GCDS-2A8	IgG1	IGHV3-11*01	95.9	2	2	**0.00276**	7	1	**0.00127**	IGHJ6*03	DGRGYNYNYNRYFYYMDV

Table 1 continued on the following page

Table 1. Continued

					FWRs			CDRs				
PBMC	Clones[1]	Isotype	V_H	Similarity	R^2	S^3	P^4(FWRs)	R	S	P(CDRs)	J_H	CDR3
	GCDS-2D11	IgG4	IGHV2-5*09	97.3	2	2	**0.04076**	4	0	**0.02137**	IGHJ4*02	RRLGLRYCSTSSCFGDFDY
	GCDS-3G9	IgG1	IGHV3-23*01	96.9	2	3	**0.00534**	6	0	**0.00340**	IGHJ4*02	DHCRSDSCYRADL
	GCDS-5D2	IgG1	ND									
	GCDS-6A6	IgG1	ND									
	GCDS-6G10	IgM	IGHV3-30*18	99.3	0	1	0.09014	1	0	0.17838	IGHJ5*02	GLLPEDIVVVPAAQTENWFDP
JLDO	JLDO-1B2	IgM	IGHV3-48*02	100.0							IGHJ4*02	DSDDYGDYGCFDY
	JLDO-2A3	IgM	IGHV3-30*03	97.0	3	1	0.08352	3	2	0.13312	IGHJ4*02	EEADFWSGYYFDY
	JLDO-3F8	IgG1	IGHV3-30-3*01	98.0	2	1	0.10667	3	0	**0.02760**	IGHJ6*03	DQQPTIYYYYYMDV
	JLDO-3G4	IgM	IGHV3-9*01	99.0	1	1	0.23217	1	0	0.26683	IGHJ6*02	DKTLYYYYGMDV
	JLDO-4B5	IgG1	IGHV3-73*02	92.6	3	6	**0.00002**	11	2	**0.00040**	IGHJ3*02	LIQVWSSQTFDI
FGS	FGS-2E5	IgM	IGHV4-59*01	100.0							IGHJ6*02	VEVKRDTGTTHPYYYGMDV
	FGS-4E4(3)	IgG4	IGHV3-48*01	94.2	5	4	**0.01181**	6	2	**0.04083**	IGHJ4*02	GRTTFGGEGQLFDY

CDR, complementary determining region; FWR, framework region; ND, not determined; PMBC, peripheral blood mononuclear cell.
[1]Numbers in parentheses indicate the identical clones isolated from the same patient. Clones with evidence of antigen selection in both FWRs and CDRs are shown in bold. Clonally related hybridomas are shown with italics.
[2]Number of replacement mutations.
[3]Number of silent mutations.
[4]*P*-value determined according to Lossos *et al.* (2000). Those values less than 0.05, which is the indication of antigen selection, are shown in bold. FWRs include FWR1, FWR2, and FWR3, whereas CDRs include CDR1 and CDR2. The *P*-values of clones with 100% similarity to germline genes are not determined.

and V_H1 gene family use decreased from 34.8 to 9.1% (χ^2, $P=0.038$). No V_H gene dominated the IgM repertoire, but IGHV3-23 may be favored in the IgG repertoire, as it was used by 5 of 22 clonally independent IgG hybridomas, although this does not reach the level of significance in comparison with IgM hybridomas (2 of 23 clonally independent hybridomas: χ^2, $P=0.1942$) (Figure 2a upper panels) or with healthy controls (12 of 71 B cells; χ^2, $P=0.5367$) (Brezinschek *et al.*, 1995). J_H use by IgM and IgG anti-DSG1 included J_H3, 4, 5, and 6, and was not significantly different between IgM and IgG hybridomas, and not different from healthy control B cells (Brezinschek *et al.*, 1995). In addition, we observed no restriction in V_H CDR3 length, as it ranged from 6 to 21 amino acids (Table 1).

We identified 11 κ and 6 λ V_L genes used by 29 sequenced clonally independent anti-DSG1 hybridomas (12 IgM and 17 IgG) (Table 2 and Figure 2b upper panels). As with V_H gene use, V_L gene use seems to be more restricted among IgG hybridomas than among IgM hybridomas. With one exception, each IgM hybridoma expressed a unique V_L gene, whereas four V_L genes were used by two or more clonally independent IgG hybridomas. IGKV1D-39 was expressed by four of 17 independent hybridomas (23.5%), suggesting that this V_L gene provides a selective advantage in binding DSG. This biased distribution of V_L is similar to that observed in responses to foreign antigens and, therefore, suggests that the anti-DSG1 B cells in FS patients undergo antigen selection.

Antigen selection of mutant anti-DSG1 B cells

The anti-DSG1 hybridomas from FS patients were extensively mutated (Table 1). The number of V_HDJ_H mutations in IgM hybridomas (166 mutations in 23 sequences; 7.2 mutations/gene) was half that in IgG hybridomas (324 mutations in 22 sequences; 14.7 mutations/gene) (χ^2, $P=0.021$). Moreover, all five unmutated sequences were from IgM hybridomas (Table 1). We used a multinomial distribution model (Lossos *et al.*, 2000) to determine bias in the frequency of amino acid replacement (R) and silent (S) mutations in framework regions (FWRs) and complementary determining regions (CDRs). We found that 5 of 23 IgM hybridomas showed a biased distribution of mutations in either FWR- or CDR-encoding regions and 1 (FS33-3H3) showed a bias distribution in both FWR- and CDR-encoding regions. In contrast, 15 of 22 independent IgG hybridomas showed a biased distribution in both FWR- and CDR-encoding regions, and most of the remaining hybridomas showed a biased distribution in either FWR- or CDR-encoding regions (Table 1). This difference between IgM and IgG hybridomas is statistically significant (χ^2, $P<0.001$). We conclude that anti-DSG B cells are antigen selected, and that mutant IgG B cells in particular have a selective advantage in contributing to this response in FS patients.

The expressed V_L genes from these hybridomas were also extensively mutated, although as is typical, V_L mutations were less frequent than V_H mutations (195 mutations in

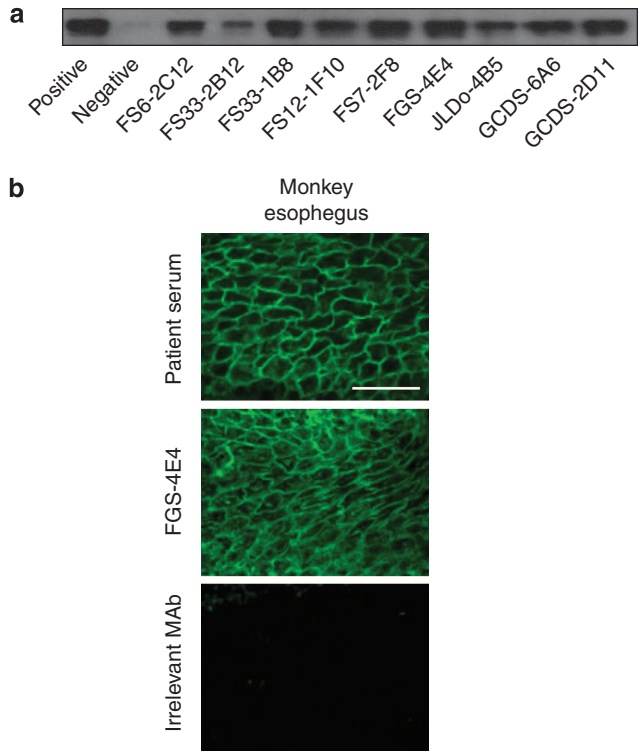


Figure 1. Anti-desmoglein 1 (DSG1) hybridoma antibodies are specific for DSG1. (**a**) Representative anti-DSG1 MAbs (all are IgG1, except FGS-4E4 and GCDS-2D11 (IgG4)) from different fogo selvagem (FS) patients were used to immunoprecipitate DSG1. Hybridoma antibodies bound to protein G agarose beads were used to immunoprecipitate His-tagged DSG1 ectodomain. The immunoprecipitated samples were used in western blots using an anti-His horseradish peroxidase conjugate to detect DSG1 protein. (**b**) Similar to serum from an FS patient, anti-DSG1 MAbs (FGS-4E4) show typical indirect immunofluorescence staining patterns using monkey esophagus as the substrate. Bar in (**b**) = 50 μm.

29 genes (6.7 mutations/gene) *vs* 490 mutations in 45 genes (10.9 mutations/gene) (χ^2, $P = 0.055$) (Table 2). In common with the V_H mutations in these hybridomas, the frequency of V_L mutation in IgM hybridomas (5.2 mutations/gene) was lower than that in IgG (7.7 mutations/gene) (χ^2, $P = 0.4434$). However, few IgG V_L genes exhibited a bias in the distribution of CDR mutations (2 of 17 V_L genes) (Table 2), suggesting that V_L mutations are less likely to improve antigen binding than V_H mutations.

Shared mutations among anti-DSG1 autoantibodies

Bias in somatic mutation was also evident by the large number of shared mutations among these hybridomas (Figure 3). For example, four of seven IGHV3-23-expressing hybridomas acquired a mutation at position 31 in the CDR1-encoding region of this V_H (Figure 3) with S31N occurring twice. In addition, CDR1 mutation S35N occurred twice and S35T occurred twice, and in CDR2, both A50S and A50G occurred twice. There were also shared mutations located in FWRs, such as N77K, Y80F, and M83I. Parallel mutations were not limited to the more frequently used IGHV3-23 and IGHV3-30 genes, as they occurred in other V_H genes (Figure 3). Interestingly, S31N occurred in three other members of the V_H1 family, and in a member each of the V_H3 and V_H5 families, further suggesting its importance to antigen binding.

Antigen selection for anti-DSG1 autoantibodies from an individual at the pre-clinical stage of disease

We have shown that healthy individuals from Limao Verde possess IgM and IgG anti-DSG1 autoantibodies (Warren *et al.*, 2000; Diaz *et al.*, 2008), strongly suggesting the existence of an environmental factor in endemic areas that sensitizes and triggers anti-DSG1 autoantibody formation in these individuals. To understand the development of anti-DSG1 antibodies in FS, we investigated the anti-DSG1 response before the onset of disease. We have collected serum samples and PBMCs from selected individuals with or without FS living in endemic regions for the past 20 years, and one non-FS individual developed FS 4 years later (patient FS45). The PBMCs of this individual, kept in liquid nitrogen, were Epstein-Barr virus transformed and fused with MSP-2S myeloma cells.

We identified 28 anti-DSG1 hybridomas (17 IgM, 11 IgG1) by ELISA (Table 3) and confirmed their specificity by immunoprecipitation (data not shown). Two clonal sets of hybridomas were identified, the largest of which consisted of six IgM hybridomas. A total of 14 V_H genes encode anti-DSG1 antibodies, but only IGHV3-23 and IGHV3-30 were used by both IgM and IgG hybridomas (Figure 2a lower panels). IGHV3-30, used by three independent FS IgM hybridomas, was the most frequently used V_H gene by pre-FS IgG hybridomas (3 of 10 clonally independent pre-FS IgG hybridomas).

The V_L gene use by pre-FS hybridomas (Table 4) was also diverse, similar to that of FS hybridomas. We identified only two V_L genes used by both pre-FS and FS hybridomas (Figure 2a lower panels and Figure 2b lower panels). The IgG hybridomas were very restricted in V_L use as five of the seven clonally independent hybridomas sequenced used IGKV2D-28 and IGKV4-1 (Figure 2a lower panel). Interestingly, we did not observe Vλ gene use among pre-FS hybridomas (0 of 17 *vs* 10 of 29 among FS hybridomas; χ^2, $P = 0.006$) (Figure 2b lower panel). Overall, although the pre-FS and FS anti-DSG1 responses overlap in V_H gene use, they exhibit notable differences in V_L gene use.

As with the FS hybridomas, the overall V_H and V_L mutation frequencies differ (266 mutations in 20 V_H genes; 59 mutations in 17 V_L genes; χ^2, $P < 0.001$). All V_H genes of pre-FS hybridomas were somatically mutated (Table 3), and IgM and IgG anti-DSG1 hybridomas exhibited similar mutation rates (123 mutations in 10 genes for IgM; 143 mutations in 10 genes for IgG; χ^2, $P = 0.7451$). These rates were also similar to those of FS IgG hybridomas (χ^2, $P = 0.6125$ and $P = 0.9404$, respectively). Multiple pre-FS hybridomas exhibited evidence of antigen selection based on biases in V_H R and S mutations. However, only 3 of 12 clonally independent IgM hybridomas showed a significant bias, and then only in FWR-encoding regions, whereas 6 of 10 clonally independent IgG hybridomas exhibited a significant bias in mutation

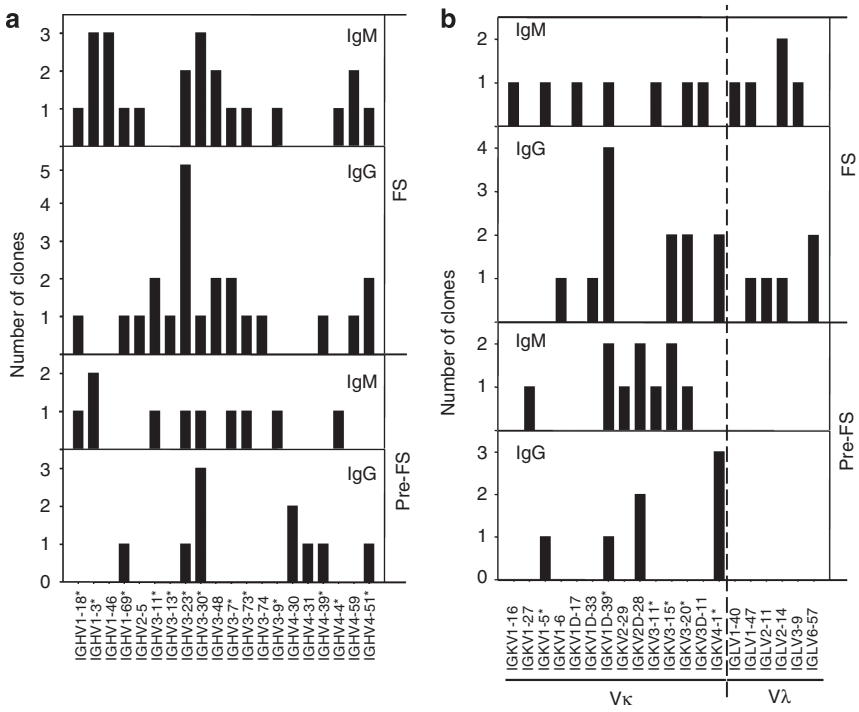


Figure 2. V gene use by fogo selvagem (FS) and pre-FS clonally independent anti-desmoglein 1 (DSG1) hybridomas. (**a**) V_H gene use. (**b**) V_L gene use. IgM and IgG are summarized separately for FS (upper panels) and pre-FS (lower panels). The number of hybridomas identified by sequence similarity to use each gene is indicated. Genes used by both FS and pre-FS hybridomas are marked by an asterisk.

in the regions encoding FWRs or CDRs, or both. Interestingly, a comparison of IGHV3-23 and IGHV3-30 sequences from pre-FS and FS hybridomas reveals multiple shared amino acid R mutations (Figure 3). The IGHV3-23 mutations A23T, S31N, S35N, A50S, A50V, S57R, and N74S, as well as IGHV3-30 mutation, S31N, occurred in both groups. In addition, the IGHV3-30 mutations, S30R and A88P, each occurred twice among pre-FS hybridomas. Thus, similar to FS hybridomas, the pre-FS anti-DSG1 hybridomas exhibit evidence of selection for mutant B cells, particularly among anti-DSG1 IgG B cells. Moreover, the shared V_H mutations suggest that the same or a similar antigen is responsible for the selective pressure in the pre-clinical and clinical stages of the disease.

DISCUSSION

In this study, we report the genetics of anti-DSG1 autoantibodies from eight FS patients and one individual 4 years before the clinical onset of FS. Our results show that the anti-DSG1 response in FS patients living in endemic regions of the disease in Brazil is antigen selected and that selection begins well in advance of the onset of clinical disease.

The hypothesis of antigen selection of anti-DSG1 B cells in FS patients is based on several lines of evidence. First, multiple groups of clonally related hybridomas were identified among hybridomas from each patient indicating that certain clones have a selective advantage in growth. Clonally related hybridoma sets are a characteristic of secondary responses to foreign antigens (Clarke *et al.*, 1985; Blier and Bothwell, 1987; Scott *et al.*, 1989) and have been observed among hybridomas derived from autoimmune patients (Qian *et al.*, 2007). Second, a limited V_H and V_L gene repertoire may be used to encode these anti-DSG1 antibodies in pre-FS and FS patients. Most V_H genes belong to V_H families 1, 3, and 4, similar to those in normal and systemic lupus erythematosus individuals (Brezinschek *et al.*, 1997; Dorner *et al.*, 1999; de Wildt *et al.*, 2000). However, IGHV3-23 is common among IgG anti-DSG1 hybridomas, increasing from 9% among IgM hybridomas to 24% among IgG hybridomas, suggesting selective pressure in favor of its use in this response. Selective V_L gene use also occurs, as we find that IGKV1D-39 increases from 8.3% among IgM hybridomas to 23.5% among IgG hybridomas. Thus, V gene use by DSG1-specific B cells may become increasingly restricted during the course of the response. Third, FS anti-DSG1 hybridomas exhibit a bias favoring the accumulation of amino acid R mutations in CDRs and S mutations in FWRs in either V_H or V_L, or both. This pattern is consistent with the selection for R mutations that provide an advantage in antigen binding (in CDRs) and against R mutations that could harm antigen binding or the structural integrity of the antibody molecule (FWRs). Consistent with this are the numerous parallel V_H mutations among these hybridomas, many of which occurred in CDRs. Taken together, the anti-DSG1 response of FS patients resembles antigen-selected responses to foreign antigen, arguing that the anti-DSG response in FS is antigen selected. This parallels our findings with the anti-DSG response in PV (Qian *et al.*, 2007).

Table 2. L chain V genes of anti-desmoglein 1 (Dsg1) hybridomas from fogo selvagem (FS) patients

					FWRs			CDRs		
PBMC	Clones[1]	Isotype	V_L	Similarity	R^2	S^3	P^4(FWRs)	R	S	*P*(CDRs)
FS6	**FS6-2C12**	IgG1	IGKV1D-39*01	92.4	8	6	**0.02417**	4	3	0.34897
	FS6-2F5(6)	IgM	IGKV3D-11*01	93.8	5	6	**0.01309**	3	2	0.35003
	FS6-3D7	IgM	IGLV3-9*01	97.9	1	2	**0.02771**	2	1	0.15775
	FS6-4A3	IgM	IGLV2-14*01	99.3	0	0	0.09251	2	0	**0.01514**
FS7	FS7-1H10	IgG2	IGKV1D-39*01	96.4	3	2	**0.03390**	2	3	0.35646
	FS7-2F8(4)	IgG1	IGKV1D-39*01	96.4	3	3	**0.03390**	3	1	0.13846
FS8	FS8-1A9	IgM	IGKV1D-39*01	96.8	1	2	**0.00465**	3	2	0.07741
FS12	FS12-1D2(2)	IgG1	IGKV3-15*01	97.6	2	1	0.11233	2	1	0.14244
	FS12-1F10(2)	IgG1	IGLV6-57*01	97.6	3	3	0.34730	0	0	0.84156
	FS12-3A7	IgG1	IGLV6-57*01	97.6	3	3	0.34730	0	0	0.84156
FS33	FS33-1B8(4)	IgM	IGKV3-11*01	98.6	0	1	**0.03299**	2	0	**0.03589**
	FS33-1D4(2)	IgM	IGLV1-40*01	99.0	0	1	0.09860	1	0	0.17658
	FS33-2E6(4)	IgG1	IGKV3-20*01	94.8	3	3	**0.00559**	4	3	0.10083
	FS33-2F4	IgG1	IGKV4-1*01	99.0	2	0	0.61661	1	0	0.29558
	FS33-3C7	IgG1	IGLV1-47*01	96.9	2	2	**0.04690**	2	2	0.28217
	FS33-4D4(2)	IgM	IGKV1-16*01	95.4	4	2	0.06498	5	0	**0.0113**
	FS33-5E11	IgM	IGLV1-47*01	100.0						
JLDO	JLDO-1B2	IgM	IGKV1D-17*01	98.9	1	0	0.20091	2	0	**0.03448**
	JLDO-2A3	IgM	IGLV2-14*03	97.9	1	1	0.44278	0	0	0.67306
	JLDO-3F8	IgG1	IGLV2-11*01	98.6	1	2	0.12676	1	0	0.33844
	JLDO-3G4	IgM	IGKV3-20*01	97.6	0	2	**0.01410**	2	0	0.07237
	JLDO-4B5	IgG1	IGKV4-1*01	97.7	0	1	**0.00818**	2	2	0.1599
GCDS	GCDS-1C2(2)	IgG1	IGKV1-6*01	96.1	4	3	0.06725	3	1	0.15831
	GCDS-2A8	IgG1	IGLV2-14*01	97.6	1	3	0.12494	0	0	0.76729
	GCDS-2D11	IgG4	IGLV6-57*01	96.5	1	3	**0.01293**	2	1	0.22925
	GCDS-3G9	IgG1	IGKV3-15*01	98.6	0	0	**0.03259**	3	0	**0.00184**
	GCDS-5D2	IgG1	IGKV3-20*01	98.3	2	2	0.21463	1	0	0.39272
	GCDS-6A6	IgG1	IGKV1D-39*01	95.8	1	5	**0.00091**	4	0	**0.03901**
	GCDS-6G10	IgM	IGKV1-5*03	98.2	1	0	**0.04899**	2	2	0.11176
FGS	**FGS-4E4(3)**	IgG4	IGKV1D-33*01	97.2	1	2	**0.01021**	2	2	0.21164

CDR, complementary determining region; FWR, framework region; PMBC, peripheral blood mononuclear cell.
[1]Numbers in parentheses indicate the identical clones isolated from the same patient. Clones with evidence of antigen selection in both FWRs and CDRs of H chains as shown in Table 1 are in bold.
[2]Number of replacement mutations.
[3]Number of silent mutations.
[4]*P*-value determined according to Lossos *et al.* (2000). Those values less than 0.05, which are the indication of antigen selection, are shown in bold. FWRs include FWR1, FWR2, and FWR3, whereas CDRs include CDR1 and CDR2.

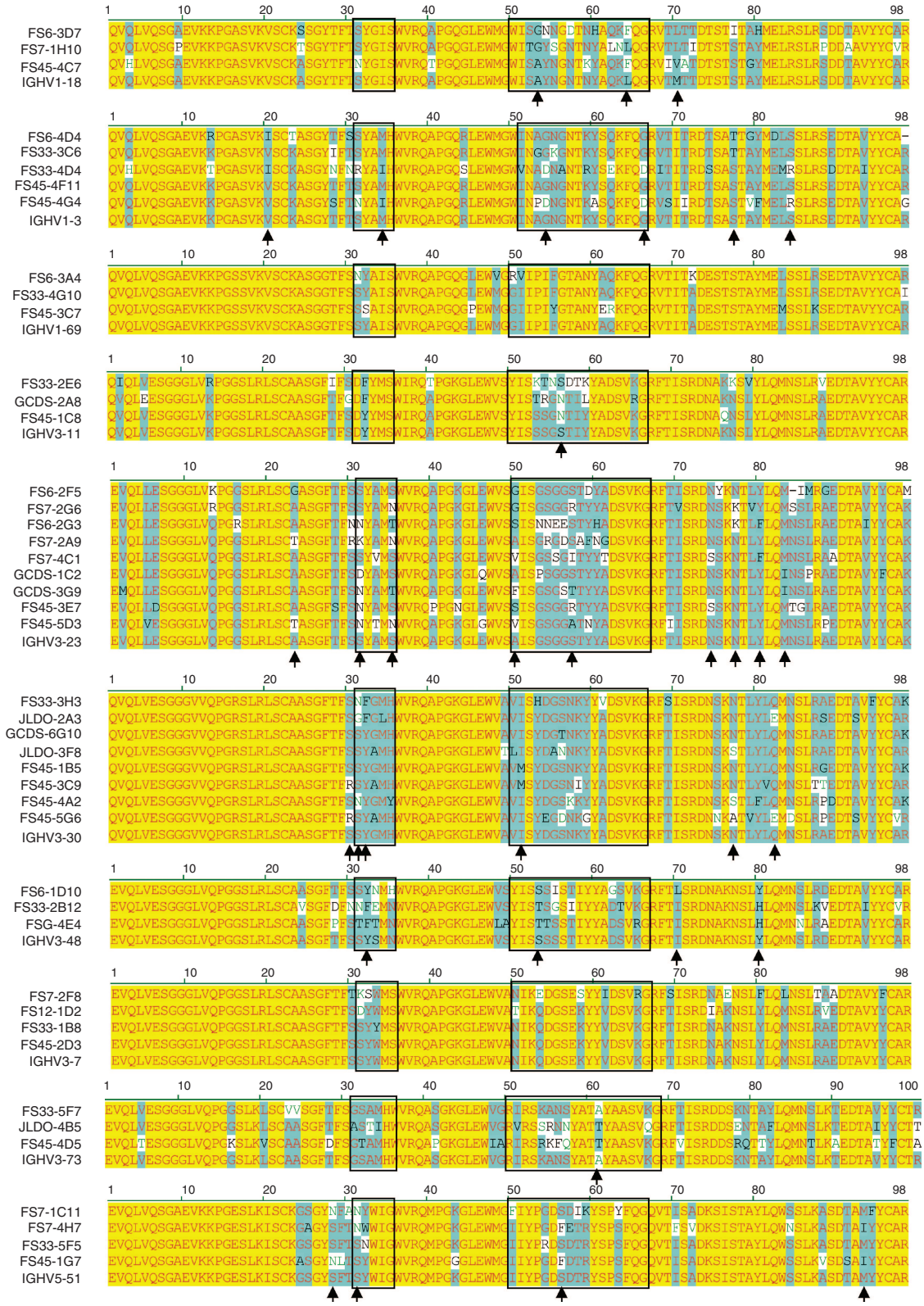


Figure 3. The expressed V_H genes from fogo selvagem (FS) and pre-FS hybridomas share replacement mutations. Sequence comparisons were conducted using Vector NTI (Invitrogen Life Technologies, Carlsbad, CA). All the sequences shown are from independent clones. Complementary determining regions (CDRs) (1 and 2) are boxed and replacement mutations are highlighted. Positions in which no mutations occurred are in yellow, whereas conservative replacement mutations are highlighted in blue and non-conservative replacement mutations highlighted in white. Positions where shared mutations occurred are indicated with arrows. Germline genes are shown at the bottom of each V_H gene set. The amino acid differences at positions 33 (G or A) and 98 (K or R) of IGHV3-30 may be polymorphisms and are not considered in this analysis.

Table 3. Anti-desmoglein 1 (Dsg1) hybridomas from an individual at preclinical stage of fogo selvagem (FS)

					FWRs			CDRs				
PBMC	Clones[1]	Isotype	V_H	Similarity	R^2	S^3	P^4(FWRs)	R	S	*P*(CDRs)	J_H	CDR3
Pre-FS	FS45-1A3	IgM	ND									
	FS45-1B5	IgM	IGHV3-30*18	99.3	1	0	0.42458	1	0	0.17838	IGHJ4*02	DRQLVGHFWTHYFDY
	FS45-1B9	IgM	IGHV4-4*07	96.6	5	1	0.33430	3	1	0.16242	IGHJ4*02	GWSFFDY
	FS45-1C10	IgG1	IGHV4-30-2*01	92.6	8	7	**0.03897**	6	1	0.15458	IGHJ6*02	GSLSAALKGCAMEV
	FS45-1C4	IgM	ND									
	FS45-1C8	IgM	IGHV3-11*01	99	1	0	**0.03869**	1	1	0.27067	IGHJ4*02	GLVQQFSYLPYYFDY
	FS45-1G7	IgG1	IGHV5-51*01	96.3	8	0	**0.00003**	1	2	0.74024	IGHJ4*02	ARVMLHLSGERTFYFDF
	FS45-2C6	IgM	IGHV3-9*01	98.6	2	1	0.38779	0	1	0.77375	IGHJ6*02	GRYSNSWYGYYSMDV
	FS45-2D3	IgM	IGHV3-7*01	99.7	0	1	0.21335	0	0	0.59333	IGHJ4*02	LGDY
	FS45-3C7	IgG1	IGHV1-69*01	96.6	3	3	**0.04427**	4	0	0.05014	IGHJ3*01	GPNRRGSYRPNDAFDI
	FS45-3C9	IgG1	IGHV3-30-3*01	96.6	4	3	0.10605	2	1	0.32406	IGHJ6*02	TSNWDGLDV
	FS45-3E7(6)	IgM	IGHV3-23*01	95.6	7	2	0.39281	3	1	0.29244	IGHJ4*02	KKAQLSAPFDY
	FS45-3H9	IgG1	IGHV4-39*06	96.6	3	1	**0.03664**	6	1	**0.00522**	IGHJ4*02	DSAERSELGYFDY
	FS45-4A2(2)	IgG1	IGHV3-30*18	96.6	4	3	0.14281	3	0	0.17202	IGHJ4*02	RAKWGSPQPYYFDY
	FS45-4C7	IgM	IGHV1-18*01	96.6	6	1	0.54624	3	0	0.17573	IGHJ6*02	DRDGYNHYYHYGMDV
	FS45-4C8	IgG1	IGHV4-31*03	96.7	5	6	0.25173	0	0	0.94877	IGHJ4*02	DLRYNYDYEGSS
	FS45-4D5	IgM	IGHV3-73*02	81.1	21	19	**0.00198**	11	5	0.37278	IGHJ4*02	ATRF
	FS45-4F11	IgM	IGHV1-3*01	99.7	0	1	0.20903	0	0	0.59112	IGHJ6*02	GGLLMTTVTTYYYYYGMDV
	FS45-4G1	IgG1	IGHV4-30-2*01	90.4	6	7	**0.00086**	11	1	**0.00191**	IGHJ4*02	AGSSYYYDSRGPIDY
	FS45-4G4	IgM	IGHV1-3*01	92.2	7	6	**0.00426**	7	3	0.07727	IGHJ3*02	ARRRKSIVVVPAARDDGFDI
	FS45-5D3	IgG1	IGHV3-23*04	93.9	4	6	**0.00153**	7	1	**0.01695**	IGHJ6*02	DSASPVLYYYYGMDV
	FS45-5G6	IgG1	IGHV3-30-3*01	94.5	4	5	**0.00518**	5	1	**0.04133**	IGHJ4*02	SVSVRLSHFDH

CDR, complementary determining region; FWR, framework region; ND, not determined; PMBC, peripheral blood mononuclear cell.
[1]Numbers in parentheses indicate the identical clones isolated. Clones with evidence of antigen selection in both FWRs and CDRs are shown in bold.
[2]Number of replacement mutations.
[3]Number of silent mutations.
[4]*P*-value determined according to Lossos *et al.* (2000). Those values less than 0.05, which are the indication of antigen selection, are shown in bold. FWRs include FWR1, FWR2, and FWR3, whereas CDRs include CDR1 and CDR2.

The endemic nature of FS, which allowed the freezing of PBMCs from individuals with a relatively high probability of developing FS, makes it possible to examine the autoreactive B-cell repertoire in humans before development of an autoimmune disease. Our analysis of one pre-FS individual indicates a remarkable similarity in pre-clinical and clinical anti-DSG1 responses. Clonal sets of hybridomas were evident among pre-FS hybridoma panels indicating uneven clonal expansion. In addition, V_H and V_L use by pre-FS and FS hybridomas overlap, particularly with the use of IGHV3-23 and IGHV3-30 (Figure 2a). There is also an overlap in Vκ use by pre-FS and FS hybridomas (6 of 14 Vκ genes, Figure 2b). Although IGKV1D-39 is expressed, it is not dominant, as it is in the FS response (Figure 2b). The most notable difference between pre-FS and FS hybridomas is the expression of Vλ. One-third (10 of 29) of the FS hybridomas, but none of the 17 pre-FS hybridomas expressed Vλ genes (Figure 2b). Unfortunately, a PBMC sample from this patient after FS diagnosis is not available. However, the analysis of anti-DSG1 antibodies in sera from this patient indicates very low levels of λ anti-DSG1 before FS onset compared with after FS diagnosis (data not shown). In contrast, there was no change in the levels of κ anti-DSG1 before and after active FS. This change in λ anti-DSG1 antibodies was observed in 4 of 11 individuals for whom sera before and after FS diagnosis were available (data not shown). This raises the possibility that λ anti-DSG antibodies are a clinical marker for FS that may predict the development of disease. We are currently testing this possibility.

The CDR replacement mutations have the most striking similarity between the pre-FS and FS hybridoma panels. A total of 18 amino-acid replacement mutations in CDR1 and CDR2 occurred in IGHV3-23 and IGHV3-30 of pre-FS and FS hybridomas. In some cases, these mutations occurred multiple times within a panel. We interpret this to mean that many of the same mutations provide a selective advantage in the clonal expansion of anti-DSG1 B cells, providing strong evidence that the same or a similar antigen is responsible for

Table 4. L chain V genes of anti-desmoglein 1 (Dsg1) hybridomas from a pre-fogo selvagem (FS) individual

					FWRs			CDRs		
PBMC	Clones[1]	Isotype	V_L	Similarity	R^2	S^3	P^4(FWRs)	R	S	*P*(CDRs)
Pre-FS	FS45-1A3	IgM	IGKV3-15*01	100.0						
	FS45-1C10	IgG1	IGKV1-5*03	95.4	3	3	**0.01783**	1	4	0.70848
	FS45-1C4	IgM	IGKV1-27*01	98.6	0	2	0.08044	0	0	0.64386
	FS45-1C8	IgM	IGKV3-15*01	99.6	0	0	0.20121	1	0	0.07719
	FS45-1G7	IgG1	IGKV1D-39*01	97.2	2	3	0.05803	2	0	0.20386
	FS45-2C6	IgM	IGKV2D-28*01	100.0						
	FS45-2D3	IgM	IGKV2-29*02	100.0						
	FS45-3C7	IgG1	IGKV4-1*01	98.3	1	3	0.06859	0	1	0.83586
	FS45-3C9	IgG1	IGKV2D-28*01	99.7	0	0	0.21961	1	0	0.09507
	FS45-3E7(3)	IgM	IGKV3-20*01	98.9	0	0	**0.03441**	3	0	**0.00223**
	FS45-3H9	IgG1	IGKV4-1*01	99.7	0	0	0.21967	1	0	0.09985
	FS45-4C7	IgM	IGKV3-11*01	99.7	0	1	0.20203	0	0	0.57947
	FS45-4D5	IgM	IGKV2D-28*01	100.0						
	FS45-4F11	IgM	IGKV1D-39*01	100.0						
	FS45-4G4	IgM	IGKV1D-39*01	95.8	2	2	**0.01459**	3	2	0.10611
	FS45-5D3	IgG1	IGKV2D-28*01	99.0	1	1	0.24701	0	1	0.73442
	FS45-5G6	IgG1	IGKV4-1*01	94.4	5	4	**0.04315**	5	1	0.11207

CDR, complementary determining region; FWR, framework region; PMBC, peripheral blood mononuclear cell.
[1]Numbers in parentheses indicate the identical clones isolated. Clones with evidence of antigen selection in both FWRs and CDRs of H chains as shown in Table 3 are in bold.
[2]Number of replacement mutations.
[3]Number of silent mutations
[4]*P*-value determined according to Lossos *et al.* (2000). Those values less than 0.05, which are the indication of antigen selection, are shown in bold. FWRs include FWR1, FWR2, and FWR3, whereas CDRs include CDR1 and CDR2.

clonal selection before and after active FS. The differences between these responses, such as Vλ gene use, may be due to targeting of different epitopes before and after active FS. Nevertheless, these data indicate that the selection of anti-DSG1 B cells begins well before the onset of clinical disease. What distinguishes between those clones that produce pathogenic autoantibodies and those that are benign or even protective has yet to be elucidated, but the current data suggest that antigen selection of mutant IgG1 anti-DSG1 B cells is not sufficient for pathogenicity, as the pre-FS individual analyzed here did not develop clinical disease for another 4 years.

The two IgG4 hybridomas overlap in V_H and V_L gene use with IgG1 hybridomas (IGHV3-48, IGHV2-5, and IGLV6-57) and, similar to IgG1 hybridomas, exhibit a bias in the distribution of mutations (Table 1). Moreover, the IgG4 hybridoma shares the CDR mutations, Y32F and S53T, with IgG1 hybridomas (Figure 3). Further analysis of more IgG4 autoantibodies is required to conclusively determine whether potential pathogenic IgG4 B cells are subject to the same or similar selective pressures *in vivo* as IgG1 B cells. Nevertheless, this study provides a view of the anti-DSG1 repertoire in FS patients indicating that the response to this self-antigen is antigen selected and begins well in advance of clinical disease. The source of the driving antigen could be environmental or self. We previously showed that patients with parasitic diseases, wherein insect bites are involved, such as onchocerciasis, leishmaniasis, and Chagas, often possess serum anti-DSG1 autoantibodies (Diaz *et al.*, 2004). Epidemiological studies of FS suggest that insect bites are a risk factor in FS (Aoki *et al.*, 2004). It has been proposed that arthropod salivary antigen(s) induces the production of cross-reactive anti-DSG1 antibodies. Alternatively, the inflammatory reaction to insect bites may expose DSG1, allowing an anti-DSG1 response.

In conclusion, we have shown that the development of autoantibodies in FS is antigen driven, similar to other autoimmune diseases, such as systemic lupus erythematosus and PV, and that antigen selection of anti-DSG1 B cells can begin years before the onset of active FS.

MATERIALS AND METHODS

Patient samples

Heparinized peripheral blood samples were collected from eight FS patients and from an individual 4 years before the onset of clinical FS. All FS patients were living in Limao Verde, Brazil, except three who were hospitalized in the Penfigo Hospital in Campo Grande, Brazil. The clinical and serological features of these patients have

been reported previously, that is, FS6 (patient no. 6), FS7 (patient no. 7), FS8 (patient no. 8), and FS 12 (patient no. 12) (Hans-Filho *et al.*, 1996). FS33 is a patient from Limao Verde who developed FS at age 44 and was not included in the previous report. The patient donating blood 4 years before the onset of disease was FS45 who developed FS at age 22 and is the son of FS12 and brother of FS46. Patient JLDO was a 23-year-old female with a 5-month history of generalized FS. Patient GCDS was a 13-year-old male with a 1-year history of generalized FS. Finally, FGS was a 33-year-old female with a 5-month history of generalized FS. All patient samples and clinical information were collected with written informed consent and adherence to the Helsinki Guidelines. All experiments were approved by the Institutional Review Boards of the University of North Carolina and the University of Sao Paulo, Brazil.

Cell preparation and hybridoma generation

Hybridomas were generated by fusion of Epstein-Barr virus-transformed PBMCs with mouse myeloma cells (P3X63Ag8.653) (Kumpel, 2000) or MFP-2S myeloma cells (Kalantarov *et al.*, 2002), as described previously (Qian *et al.*, 2007). MFP-2S myeloma cells were kindly provided by Drs Kalantarov and Trakht from Columbia University, New York, USA. Hybridomas secreting anti-DSG1 antibodies were screened using a DSG1-specific ELISA, as described previously (Warren *et al.*, 2000, 2003; Li *et al.*, 2003; Diaz *et al.*, 2004). The specificity of these autoantibodies was confirmed by immunoprecipitation (Warren *et al.*, 2000; Li *et al.*, 2003) and indirect immunofluorescence using monkey esophagus (IF) as described (Anhalt *et al.*, 1982; Ding *et al.*, 1997).

Sequence analysis of V_H and V_L region from cloned hybridoma cells

Messenger RNA isolation, PCR amplification, and sequence analysis were conducted as described previously (Qian *et al.*, 2007). The distribution of somatic mutations was analyzed according to the multinomial distribution model established by Lossos *et al.* (2000) and the *P*-values were calculated using the JAVA applet at http://www-stat.stanford.edu/immunoglobin. A *P*-value of less than 0.05 is taken as evidence for antigen selection at FWR and CDR (Lossos *et al.*, 2000).

CONFLICT OF INTEREST

The authors state no conflict of interests.

ACKNOWLEDGMENTS

This work was supported in part by the US Public Health Service Grants R01-AR30281, RO1-AR32599, and T32 AR07369 awarded to L.A.D., R01-AI43587 to S.H.C, and a Dermatology Foundation Research Grant and an American Skin Association Alice P. Melly Research Grant to Y.Q.

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