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Dissecting the Anti-Desmoglein Autoreactive B Cell Repertoire in Pemphigus Vulgaris Patients[1]

Ye Qian,* Luis A. Diaz,[2]* Jian Ye,‡ and Stephen H. Clarke‡

Pemphigus vulgaris (PV) encompasses two clinical phenotypes, one producing mucosal blisters and the other mucosal and skin lesions (mcPV). The mucosal blister-producing PV variant is characterized by autoantibodies against desmoglein (Dsg)3, whereas mucosal and skin lesion-producing PV is characterized by autoantibodies to Dsg3 and Dsg1. The present study was aimed at disclosing the diversity and clonality of the anti-Dsg3 response, as well as whether anti-Dsg3 B cells are Ag selected. Human-mouse heterohybridomas were generated by fusion of EBV-transformed or freshly isolated PBLs from six PV patients with mouse myeloma cells. A total of 73 anti-Dsg hybridomas (47 IgM and 26 IgG) were isolated. Over 90% are specific for both Dsg1 and Dsg3 indicating extensive cross-reactivity between these responses. V_H gene segment use by IgM hybridomas is diverse, but is restricted among IgG hybridomas, where the majority uses one of two V_H genes. V_L gene segment use was diverse even among IgG hybridomas suggesting that the V_L is less critical to defining desmoglein specificity. Additionally, the IgG hybridomas were extensively mutated and the distribution and nature of the mutations suggested that they had been Ag selected. We conclude that the potentially pathogenic IgG anti-Dsg response is restricted in V_H use, is somatically mutated, and is Ag selected. ***The Journal of Immunology,*** **2007, 178: 5982–5990.**

Pemphigus vulgaris (PV),[3] lethal if untreated, is a human organ-specific autoimmune mucocutaneous disease mediated by pathogenic IgG autoantibodies (1). These autoantibodies recognize the ectodomain of two desmosomal glycoproteins, desmogleins (Dsg) 3 and 1 (2). Dsg3 and Dsg1 are glycoproteins that belong to the cadherin superfamily of calcium-dependent cell adhesion transmembrane molecules (3) and are important for the integrity of the epidermis. PV encompasses two clinical phenotypes, one producing mucosal blisters (mPV) and the other mucosal and skin lesions (mcPV) (4). The mPV variant is characterized by autoantibodies against Dsg3, whereas the mcPV form produces anti-Dsg1 and anti-Dsg3 Abs (4, 5), although transition from one to another may occur. These antigenic molecules belong to the desmosomal subfamily of cadherins, a group of calcium-dependent cell adhesion glycoproteins (6, 7), that are crucial mediators of squamous epithelium cell adhesion (7). It is generally accepted that anti-Dsg3 autoantibodies are the hallmark of PV (6) and anti-Dsg1 autoantibodies are the serological markers of pemphigus foliaceus (PF), a closely related epidermal-specific autoimmune blistering disease of the skin (8). Although rare, clinical and serological transitions from PV to PF (9) and PF to PV (10) have been reported. Moreover, we have demonstrated that autoantibodies against Dsg3 and Dsg1 from mcPV patients are Ag specific and pathogenic (11). It is feasible, however, that PV patients also possess cross-reactive autoantibodies due to the extensive sequence homology of Dsg3 and Dsg1, especially at the ectodomain level (6). Recent studies by Payne et al. (12) appear to support this latter notion because they demonstrated the existence of autoantibodies with single specificity to either Dsg1 or Dsg3 and double specificities to both Dsg1 and Dsg3 in clones derived from one mcPV patient.

Pathogenic PV autoantibodies are predominantly IgG4 (13–15) and recognize epitopes located on the ectodomain of Dsg3 (16). Importantly, not only whole IgG, but also $F(ab')_2$ and Fab of PV IgG, are pathogenic when passively transferred into neonatal mice, where they reproduce the key features of the human disease in animals (1, 17). It is hypothesized that PV autoantibodies following their binding to Dsg3 induce epidermal cell detachment (named acantholysis), which leads to blisters and erosions in the skin and mucosas. Recent studies suggest that PV autoantibodies may activate intracellular signaling pathways (18, 19) that lead to apoptosis and cell detachment (20, 21).

Unlike other autoimmune diseases such as systemic lupus erythematosus (22) and rheumatoid arthritis (23) where the autoantibody repertoires have been extensively studied, these investigations in PV are very limited or absent. One of these studies was recently published by Payne et al. (12) where they cloned Dsg1 and Dgs3 mAbs from a mcPV patient using phage display technology. They have demonstrated that the H and L chain usage in a PV patient is restricted, but were unable to distinguish the isotype repertoires or to determine in vivo V_H/V_L association. These results may have had an inherent technique bias during the PCR-based capture of V_H and V_L gene segments, although some of the isolated mAbs were pathogenic by passive transfer experiment.

Genetic analysis of the V region genes of human autoantibodies not only provides invaluable information to the study of the etiology and pathogenesis of autoimmune diseases, but also makes possible the generation of anti-idiotypic Abs that may be clinically

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[3] Abbreviations used in this paper: PV, pemphigus vulgaris; Dsg, desmoglein; mPV, PV producing mucosal blisters; mcPV, PV producing mucosal and skin lesions; PF, pemphigus foliaceus; PB, peripheral blood; RT, reverse transcriptase; EC, ectodomain; FS, fogo selvagem; FWR, framework region; R, replacement; S, silent.

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applicable to treat these diseases. Thus, by determining the V region sequence of pathogenic autoantibodies it would be possible to specifically target pathogenic B cells or pathogenic autoantibodies. These possibilities may benefit PV patients, who like patients with other autoimmune diseases, are controlled by systemic steroid and immunosuppressive therapy. These therapies are associated with devastating complications such as osteoporosis, diabetes, hypertension, infections, etc. (24).

The purpose of this study was to isolate anti-Dsg3- and anti-Dsg1-specific B cells from PV patients to clone and sequence the V region genes of the respective anti-Dsg3 and anti-Dsg1 autoantibodies. Human-mouse heterohybridomas were generated by fusion of mouse myeloma cells with EBV-transformed or freshly isolated PBLs from six PV patients. Screening was conducted using recombinant Dsg1 and Dsg3 ELISA as reported previously (25, 26), and cloning by limiting dilution. A total of 73 hybridomas were isolated (47 producing IgM and 26 IgG). Over 90% of the hybridomas produced Abs that recognize both Dsg1 and Dsg3, indicating cross-reactivity between these Ags. The V(D)J gene segments used by IgM hybridomas were diverse, whereas IgG-producing hybridomas showed a considerable restriction with the majority of them using either of two V_H gene segments and a single J_H segment. The V_L gene segments used were diverse even among IgG hybridomas, suggesting that the V_L is less critical to defining desmoglein specificity. Additionally, the cross-reactive IgG anti-Dsg3/Dsg1 Abs were more extensively mutated and showed evidence of Ag selection. We conclude that the potentially pathogenic IgG anti-Dsg response in human PV is restricted in V_H use, somatically mutated, and the product of Ag selection.

Materials and Methods

Patients' samples

Heparinized peripheral blood (PB) samples were collected from six PV patients. Two patients presented mPV (patients 1 and 6) and the remaining presented the mcPV form. The patients' sera showed pemphigus autoantibodies in titers ranging from 1:80 to 1:5120 by indirect immunofluorescence techniques (1, 4). These autoantibodies recognized Dsg3 and Dsg1 by immunoprecipitation and ELISA (25–27). Human keratinocytes were grown in culture and used to test mAbs by indirect immunofluorescence techniques as described by Hu et al. (28).

Cell preparation and cell fusion

Hybridomas were generated by fusion of either freshly isolated PBMCs or EBV-transformed PBMC with mouse myeloma cells (P3X63Ag8.653) (29). The hybridomas obtained from PV-4 and PV-6 were from direct fusion of fresh PBL with myeloma cells, whereas hybridomas from the rest of the patients were from fusion of EBV-transformed B cells with myeloma cells. Fresh PBMCs were purified from heparinized PB samples (~20 ml) by Ficoll centrifugation. The buffy coat was washed twice in PBS and subjected to fusion with myeloma cells. EBV transformation of patient B cells was conducted by the University of North Carolina Tissue Culture Facility by standard procedures (30). Fused heterohybridomas were cultured in RPMI 1640 with 20% FCS and hybridoma-cloning factors (BioVeris) plus hypoxanthine aminopterin thymidine and 10 μM ouabain in 96-well plates. After 2–3 wk, culture supernatants were screened using Dsg1- and/or Dsg3-specific ELISAs (25, 26). Dsg1- or Dsg3-positive hybridomas were cloned limiting dilution.

ELISA

For Dsg1- and Dsg3-specific ELISAs, ELISA plates (Nunc) were coated with baculovirus-expressed Dsg1 or Dsg3, and the ELISA was conducted according the protocol described previously (25, 26). To determine the isotype of the mAbs, ELISA plates were coated with either mouse anti-human IgM (SA-DA4), or IgG (JDC-10; Southern Biotechnology Associates). To determine the subclass of IgG-producing mAbs, the plates were coated with mouse anti-human IgG1, IgG2, IgG3, or IgG4 mAbs (Zymed Laboratories). Peroxidase-conjugated mouse anti-human IgG or rabbit anti-human IgG plus IgM Abs (Jackson ImmunoResearch Laboratories) were added and the plates were developed using the TMB Microwell Peroxidase substrate system (KPL) for 20 min with 100 μl/well, and stopped with 50 μl/well 4 N H_2SO_4 and read at 450 nm.

Table I. *Anti-Dsg3 hybridomas isolated from PV patients and healthy individuals*

	PV Patients	Healthy Donors
Fusion wells[a]	657	115
% Growth-positive wells[b]	100	100
No. of anti-Dsg3-positive wells[c]	73	3

[a] Fusion wells: total number of wells tested.
[b] The percentage of tested wells with growing hybridomas.
[c] The number of wells positive for anti-Dsg3 Abs by ELISA.

Amplification of V_H and V_L region from cloned hybridoma cells

Approximately 100-1000 cloned hybridoma cells from 96-well tissue culture plates were collected and mRNA isolated using TurboCapture 8 mRNA kit (Qiagen) according to the manufacturer's instructions. The V_H and V_L region sequences from each hybridoma cell line were obtained by RT-PCR. Fifty microliters of reverse transcription (RT) reaction mix (10 μl of 5× RT buffer, 2.5 μl of 10 mM dNTP mix, 5 μl of 0.1 mM DTT, 1.5 μl of RT Superscript II (Invitrogen Life Technologies), 1 μl of each C region primer (CμI, CγI, CκI, and CλI) (31) and 27 μl of diethyl pyrocarbonate-treated H_2O) was added per tube and the strip was incubated for 50 min at 42°C followed by 10 min at 70°C. A total of 50 μl of PCR mix (5 μl of 10× PCR buffer, 5 μl of 2 mM dNTP mix, 1 μl of $MgCl_2$, 1 μl of *Taq*, 1.6 μl of primer mix, and 36.4 μl of distilled H_2O) was then added per well. Oligonucleotide mixtures were used as reported by Wang and Stollar (31), only the primers for the second round of PCR were modified by removal of the restriction sites. Touchdown PCR (32) was used for amplification of *V* genes and run for 35 cycles. Denaturation was set at 98°C for 45 s and elongation at 72°C for 1 min. The annealing was set at each cycle for 45 s while the temperature varied over the course of the PCR. The annealing temperature of the first two cycles of the touchdown PCR cycle was 65°C and the temperature was decreased at every other cycle by 2°C until a temperature of 55°C was reached. The remaining 25 cycles were run at 55°C. In cases where the first PCR failed to yield a

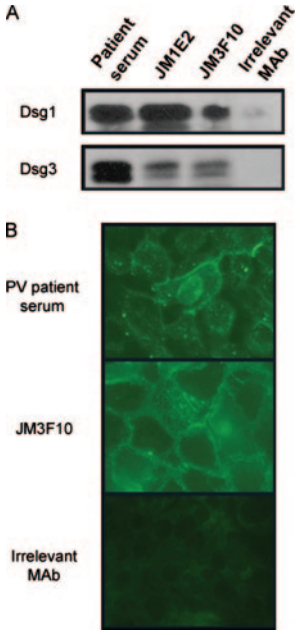

FIGURE 1. Human mAbs produced by anti-Dsg hybridomas recognize both Dsg1 and Dsg3 Ags by immunoprecipitation and immunofluorescence analysis. *A*, Immunoprecipitation of Dsg1 and Dsg3 by IgG mAbs (11, 27). *B*, IgG anti-Dsg mAb (JM3F10) bind the cell surface of human keratinocyte in culture producing the same pattern as PV serum. Control irrelevant mAb shows negative staining. Human keratinocytes were stained as described as described by Hu et al. (28). The mAbs produced by our hybridomas fail to stain cryosections of human skin (data not included).

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Table II. *Dsg-specific hybridomas from PV patients identified by ELISA*

			Dsg[b]		V_H				V_L		
Patient	Clones[a]	Isotype	1	3	Germline	Mutations[c]	J_H	CDR3	Germline	Mutations[c]	J_L
PV-1	EF1A8	IgM	+	+	IGHV3-23*01	1/0	J_H6	TSYYYGMDV	IGKV1-17*01	0/0	Jκ2
	EF1A10	IgM	−	+	IGHV3-30*18	0/0	J_H4	DQAVAGIGTVDY	IGKV3-11*01	0/0	Jκ4
	EF1B9	IgM	+	+	IGHV3-66*01	0/0	J_H6	GGFWSGYFGV	IGKV1-16*01	1/0	Jκ4
	EF1D3	IgM	+	+	IGHV1-2*02	6/2	J_H3	GKRQFDAFDI	IGKV1D-39*01	1/0	Jκ1
	EF1E11	IgM	+	+	IGHV3-23*01	0/0	J_H4	PTNWWLRECRFDY	IGKV1-16*01	2/0	Jκ4
	EF1F11	IgM	−	+	IGHV2-70*01	2/1	J_H6	SYGIGFYYSYGMDV	IGLV1-44*01	1/0	Jλ3
	EF2B8	IgG1	+	+	IGHV3-30*18	4/3	J_H4	QGIAVAFDS	IGKV3-20*01	4/1	Jκ4
	EF2E9	IgG1	+	+	IGHV3-30*18	9/8	J_H5	DGRGVTMVQGVISRGWFDP	IGKV2D-40*01	3/1	Jκ5
	EF2F7	IgM	+	+	IGHV3-23*01	9/8	J_H4	YPTSNTWYGESYYFDH	ND		
	EF2G2	IgM	+	+	IGHV4-61*02	0/0	J_H4	EGMGRFSRRPAAGGALN	IGLV1-44*01	0/0	Jκ2
	EF3B10	IgM	−	+	IGHV2-26*02	0/0	J_H4	RWYSGSYLDSYYFDY	ND		
	EF3B3	IgM	+	+	IGHV4-61*02	0/0	J_H4	GSPTCSSTSCYRRYYFDY	IGKV1D-39*01	0/0	Jκ2
	EF3B6	IgM	+	+	IGHV3-53*01	21/8	J_H4	STKWTGYFDY	IGKV4-1*01	5/4	Jκ1
	EF3E2	IgM	+	+	IGHV3-30-3*01	5/1	J_H6	GGWLRLLGGMDV	IGLV2-8*01	3/0	Jλ2
	EF3E8	IgM	+	+	IGHV3-30-3*01	3/1	J_H4	DAGDYCSGGSCHRGDYFDY	ND		
	EF4A6	IgG1	+	+	IGHV3-33*01	13/6	J_H4	GGTIFGVVVVEKNQFDY	ND		
	EF4C6	IgM	+	+	IGHV1-69*01	0/0	J_H3	PGHLVANGDAFDI	IGKV2-24*01	0/0	Jκ4
	EF4E11	IgM	+	+	IGHV3-33*01	0/1	J_H6	ATEDLWCMDV	ND		
	EF4E5	IgM	+	+	IGHV3-43*01	0/0	J_H6	DYIPLRGYSGYDATNRFDP	IGLV1-47*01	0/0	Jλ3
	EF4G11	IgM	+	+	IGHV4-4*02	0/0	J_H3	GKGDELLSIAVAGTHAFDI	IGKV1-5*03	5/2	Jκ4
	EF4G12	IgM	+	+	IGHV3-33*01	2/3	J_H5	ASYHILNGFET	IGLV8-61*01	1/3	Jλ3
	EF4H6	IgM	+	+	IGHV3-53*01	6/8	J_H3	NKHDSSGPHNAFDI	IGKV1D-16*01	4/0	Jκ4
	EF5E12	IgM	+	+	IGHV4-30-4*01	0/0	J_H6	DFYDFWSGYSLYGMDV	IGKV1-17*01	0/0	Jκ1
	EF5F12	IgM	+	+	ND				IGKV1-5*03[d]	0/0	Jκ2
	EF5H8(2)[e]	IgM	+	+	IGHV4-4*02	0/2	J_H4	**SGFLDDNTLYQGIDY**	IGKV1-5*03[d]	4/1	Jκ2
PV-2	**WE1C6**	IgG1	+	+	IGHV3-30*13	17/12	J_H4	**DLSGAATRYLES**	IGKV1-12*02	10/5	Jκ5
	WE3H9	IgG1	+	+	IGHV3-30*13	17/14	J_H4	**DLSGAATRYLES**	IGKV1-12*02	10/5	Jκ5
	WE1E10(4)	IgM	+	+	IGHV3-30*18	6/3	J_H6	*ERTVATLYHYYYYGMDV*	IGLV1-44*01	5/2	Jλ2
	WE2D6(3)	IgM	+	+	IGHV3-72*01	13/5	J_H6	GADLSKYYYDSSGSGYYYGMDV	IGKV1-5*03	4/2	Jκ4
	WE3A2	IgM	+	+	IGHV3-72*01	12/6	J_H6	GADLSKYYYDSSGSGYYYGMDV	IGKV1-5*03	4/2	Jκ4
	WE4G2(2)	IgM	+	+	IGHV3-72*01	12/5	J_H6	GADLSKYYYDSSGSGYYYGMDV	IGKV1-5*03	4/2	Jκ4
	WE2F6	IgM	+	+	ND				IGLV2-14*01	8/5	Jλ2
	WE4C8	IgM	+	+	IGHV4-34*02	5/1	J_H6	LRPWYQQRYYYGMDV	ND		
	WE5D10	IgG1	−	+	IGHV3-23*01	3/3	J_H4	PRWELLARPYFDY	IGKV1D-39*01	5/1	Jκ1
	WE5G4	IgG1	+	+	IGHV3-30*18	1/1	J_H6	DGWGYCSSTRCLNWFDP	IGKV3-15*01	5/0	Jκ4
	WE5H5	IgM	+	+	IGHV3-23*01	7/4	J_H4	YLVVAGLRYHFDS	IGKV4-1*01	3/0	Jκ4
PV-3	JB1D10	IgG1	+	+	IGHV3-23*01	10/0	J_H3	DPLDITMVRGIIRRQGGSA	IGKV1-5*01	4/3	Jκ1
	JB1D1(2)	IgM	+	+	IGHV3-23*01	4/8	J_H4	**DMEQWLVRGTWWY**	ND		
	JB1F7	IgG2	+	+	IGHV3-30*14	15/7	J_H6	VKYCRGGSCPGGMDV	IGKV1D-39*01	9/6	Jκ4
	JB2E1	IgG1	+	+	IGHV4-61*01	24/5	J_H4	AWQLPYTFDF	IGKV4-1*01	6/5	Jκ4
	JB2G3	IgM	−	+	IGHV3-33*04	10/3	J_H4	DRSSSYFDH	IGLV2-11*01	2/1	Jλ3
	JB3A1	IgM	+	+	IGHV3-33*05	6/1	J_H2	IRGGHSSSWYFDY	IGLV1-51*01	4/1	Jλ2
	JB3F10	IgG1	+	+	IGHV3-30*01	2/1	J_H4	DGARSPKFMVVATIRGYFDY	ND		
	JB5A2	IgM	+	+	IGHV3-23*01	11/6	J_H4	QAVGYSYNSFDY	IGKV1D-39*01	10/6	Jκ4
	JB5E3	IgM	+	+	IGHV4-31*03	0/0	J_H6	SRKLDSAVIKIYYYYYGMDV	IGKV1-5*01	0/0	Jκ1
PV-4	J2A6	IgM	+	+	IGHV1-69*06	0/0	J_H6	LGYYGDYEGRRRNYYYYGMDV	IGKV3-11*01	0/0	Jκ4
	J5B12	IgM	+	+	IGHV4-30-2*01	4/5	J_H5	GFQPYGDYYSKLPKTNGST	ND		
	J4F12	IgG1	+	+	ND				IGKV1-5*03	3/3	Jκ1
PV-5	**JM1B11(8)**	IgG1	+	+	IGHV3-23*01	5/2	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	2/3	Jλ3
	JM1C5	IgG1	+	+	IGHV3-23*01	5/3	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	2/3	Jλ3
	JM1E2	IgG1	+	+	IGHV3-23*01	4/3	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	2/3	Jλ3
	JM1E11	IgG1	+	+	IGHV3-23*01	6/2	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	2/3	Jλ3
	JM3F10	IgG1	+	+	IGHV3-23*01	7/2	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	2/3	Jλ3
	JM3E5	IgG1	+	+	IGHV3-23*01	7/3	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7-46*01	3/4	Jλ3
	JM3E3	IgG1	+	+	IGHV3-23*01	7/3	J_H4	**FWAPYCGSTSCYTPFDY**	IGLV7–46*01	2/3	Jλ3
PV-6	CG2D12	IgM	+	+	IGHV3-30-3*01	0/0	J_H4	ARCGGSCYMGLYYFDY	IGLV1-51*01[f]	0/0	Jλ2
	CG4E6	IgM	+	+	ND				IGLV1-51*01[f]	0/0	Jλ2
	CG6E4	IgM	+	+	IGHV3-9*01	0/0	J_H3	YYCAKDREWELLRAFDI	IGKV3-20*01	0/0	Jκ3

[a] Numbers in parentheses indicate the identical clones isolated from the same patient.
[b] The reactivities of Abs produced by hybridomas to Dsg1 or Dsg3 as determined by Dsg1- or Dsg3-specific ELISA.
[c] Total replacement mutation and silent mutations (R mutation/S mutations) on FWR1,2,3 and CDR1,2 are shown.
[d] They are clonally independent hybridomas as the L chain CDR sequences of this pair are different.
[e] Clonally related hybridomas are shown with bold, italics, or underlining. Multiple clones from the same patient are distinguished in the same manner, with bold, italics, or underlining.
[f] They are clonally independent hybridomas as the L chain CDR sequences of this pair are different.

product, the second round of PCR was conducted using the touchdown PCR described above. The products of the PCR were analyzed on a 2% agarose gel. PCR products were cleaned by ExoSAP-IT (Amersham Biosciences). DNA was sequenced using the PCR 3′ and 5′ primers by the Automated DNA Sequencing Facility at UNC. V_H and V_L genes were identified using IgBlast (www.ncbi.nlm.nih.gov/igblast/) and IMGT (http://imgt.cines.fr/textes/IMGTrepertoire/). Domain classification is according to Kabat et al. (33).

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Table III. *CDR3 nucleotide sequences of clonally related hybridomas*

Hybridomas[a]	CDR3				
	V[b]	N	D	N	J
EF5H8(2)	T	CGGGGTTTCTCGACGATAACACTCTATACC	AGGGTA(TTG)[c]		ACTAC
WE1C6	GA	TTTGTC	GGGAGCTGCCAC	CCG	CTACCTTGAGAGC
WE3H9	--	------	----------	---	----------
WE1E10(4)	GA	GCGGAC	AGTGGCTAC	CCTAT	ATCACTACTACTACTATGGTATGGACGTC
WE2D6(3)	G	GGGCTGATTTGAGCAA	GTATTATTATGACAGTAGTGG	CTCGGG	CTATTATTACGGTATGGACGTC
WE3A2	-	----------	----------	------	----------
WE4G2(2)	-	----------	----------	------	----------
JB1D1(2)	GA	CATGG	AGCAGTGGCTGGT	TAGGGGGACTTGGTGG	TAC
JM1B11(8)		CTTCTGGGCACC	ATATTGTGGTAGTACCAGCTGCTATAC	GCC	CTTTGACTAC
JM1C5		----------	----------	---	----------
JM1E2		----------	----------	---	----------
JM1E11		----------	----------	---	----------
JM3F10		----------	----------	---	----------
JM3E5		----------	----------	---	----------
JM3E3		----------	----------	---	----------

[a] Numbers in parentheses indicate the number of sequences with the identical sequence throughout the V region segment.
[b] Nucleotides for the V, D, and J segments, and nucleotide region are indicated as V, D, J, and N, respectively.
[c] The nucleotides that appear in parentheses are overlapping between D and J and therefore can be of either D or J segment origin.

Results

Isolation of anti-Dsg autoantibody producing B cells by generation of heterohybridomas

To study the anti-Dsg response in PV patients, EBV-transformed or freshly isolated PB B cells from six patients were fused with mouse myeloma cells to generate heterohybridomas. Hybridomas were screened for anti-Dsg reactivity by a Dsg-specific ELISA (25, 26) and subcloned. A total of 73 hybridomas were subcloned and analyzed in this study (Table I). Approximately two-thirds (47 of 73) of the hybridomas isolated from PV patients were IgM and one-third (26 of 73) were IgG with nearly all (25 of 26) of the latter IgG1. These 73 Dsg-positive hybridomas were derived from a screen of 657 wells (Table I), each of which contained between 1 and 10 independently fused hybridomas. In contrast, only 3 Dsg-positive hybridomas were identified among 115 wells (1–10 hybridomas/well) generated by fusion of EBV-transformed B cells from two healthy individuals (Table I). Thus, the frequency of anti-Dsg hybridomas in PV patients is significantly higher than in healthy controls ($p = 0.0085$; χ^2 test) consistent with an expansion of the anti-Dsg B cell repertoire due to autoimmunity.

Nearly all hybridomas (67 of 73) were specific for both Dsg1 and Dsg3, as determined by Ag-specific ELISAs (25, 26), indicating that most anti-Dsg Abs are cross-reactive. Specificity was confirmed by immunoprecipitation and immunofluorescence analysis of selected hybridoma Abs. These Abs precipitated recombinant Dsg1 and Dsg3 from Hi 5 cell culture supernatants infected by Dsg-producing baculoviruses (Fig. 1*A*) and, as is characteristic of serum anti-Dsg Abs, exhibit membrane staining of cultured human keratinocytes (Fig. 1*B*).

Evidence of oligoclonality

To assess the diversity of the anti-Dsg response in these patients, the expressed V_H and V_L segments were amplified by RT-PCR of isolated mRNA from each hybridoma and sequenced. The involvement of a limited number of B cell clones is a hallmark of secondary IgG responses to foreign Ags by both humans (34) and mice (35, 36), and is due to Ag selection favoring the expansion of some clones over others. It is also a hallmark of autoantigen-specific responses in mouse (37–39) and has been seen in human autoimmunity (40). Hybridomas derived from B cells of the same clone will have identical V(D)J junctional sequences, whereas, due to the extensive diversity possible at the V-D and D-J junctions, those derived from different clones will invariably have different junctional sequences. By these criteria, our sequence analysis reveals six sets of clonally related hybridomas from four of the six patients, one from PV-1, three from PV-2, one from PV-3, and one from PV-5 (Tables II and III). All but two of these clonal sets were IgM hybridomas (Table II). Interestingly, the clonal set from PV-5 included all 14 of the IgG1 hybridomas isolated from this patient. Thus, the autoimmune response to Dsg 1 and 3 exhibits oligoclonality consistent with Ag selection.

V_H gene segment use by PV Abs

The identity of the V(D)J gene segments expressed by these hybridomas was determined by comparison to germline sequences

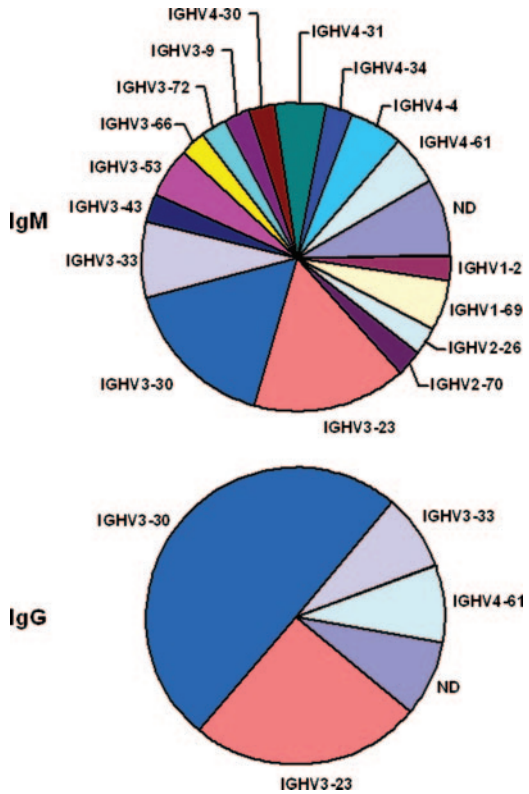

FIGURE 2. V_H gene segment use by IgG anti-Dsg hybridomas was more restricted than V_H gene use by IgM anti-Dsg hybridomas. A total of 17 V_H gene segments were identified among anti-Dsg IgM Abs (*left*), while only 4 different V_H gene segments were identified among anti-Dsg IgG hybridomas (*right*). Each V_H gene segment is identified by IMGT (http://imgt.cines.fr/textes/IMGTrepertoire/) nomenclature.

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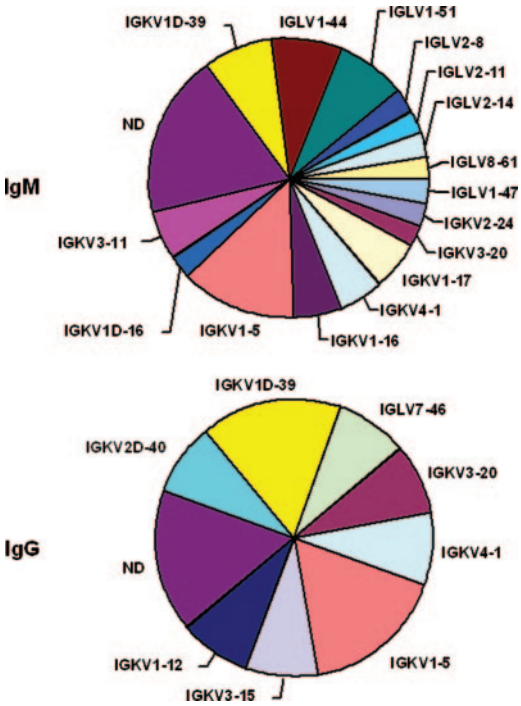

FIGURE 3. V_L gene use by both IgM and IgG anti-Dsg hybridomas was extensive. A total of 16 different V_L genes were identified among the IgM hybridomas, while 8 were identified among IgG hybridomas. This difference may be due to the larger IgM sample size. Each V_L gene is identified by IMGT (http://imgt.cines.fr/textes/IMGTrepertoire/) nomenclature.

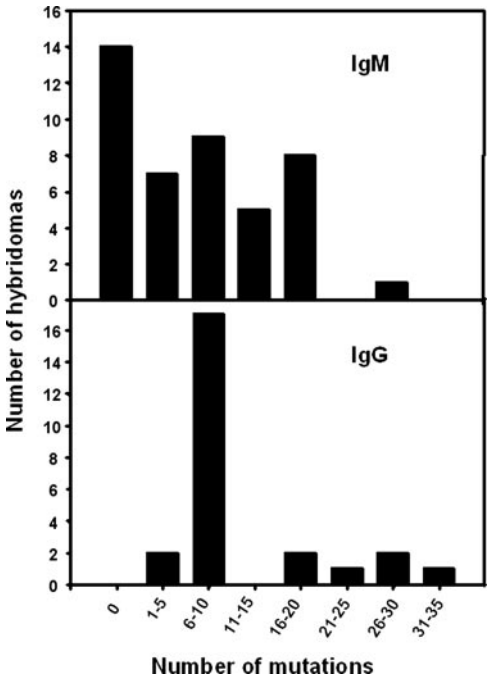

FIGURE 4. IgG anti-Dsg hybridomas are more extensively mutated than IgM anti-Dsg hybridomas. Mutations were identified by comparison to germline sequences. Hybridomas were clustered by a defined range of mutations and plotted. Both clonally related and unrelated hybridomas were included, because clonally related hybridomas often differ in mutations.

(IgBlast and IMGT) (Table II). A total of 17 V_H genes were identified from the 45 clonally independent hybridomas sequenced for H chain. Most of this diversity was among the IgM hybridomas. IgM hybridomas used a total of 17 different V_H gene segments, while IgG hybridomas used only 4 (Table II and Fig. 2). Moreover, two V_H gene segments, *IGHV3-23* and *IGHV3-30*, were used by 9 of 11 clonally independent IgG hybridomas sequenced, compared with 11 of 34 IgM hybridomas ($p = 0.0041$; χ^2 test). This bias for *IGHV3-23* and *IGHV3-30* use by IgG hybridomas extended to individual patients, as IgG hybridomas expressing these genes were identified in all four patients for which IgG sequences were available. J_H gene segment use by IgM hybridomas was also biased. J_H4 and J_H6 were used by 26 of 34 clonally independent IgM hybridomas (Table II), but this is similar to that observed among healthy control naive B cells (41) and therefore not indicative of Ag selection. In contrast, J_H4 is used by 7 of 11 clonally independent IgG hybridomas compared with 14of 34 for IgM (Table II), although this difference does not reach statistical significance, probably due to the small IgG sample size ($p = 0.19$; χ^2 test). There was no apparent restriction in CDR3 length, as it varies in length from 9 to 22 aa among IgM and 9–20 among IgG hybridomas (Table II). Thus, IgG, but not IgM, anti-Dsg H chains exhibit restrictions in V_H and possibly J_H use, suggesting that the former have been Ag selected.

V_L *gene segment use by PV Abs*

Sequence analysis of the V_L-J_L rearrangements revealed a total of 21 V_L genes used to encode anti-Dsg-autoantibodies (Table II and Fig. 3). Seventy percent (38 of 40) of clonally independent anti-Dsg hybridomas used κ L chain genes and 30% (12 of 40) λ L chain genes (Table II). As with V_H use, some V_L genes were used by multiple clonally independent hybridomas. For example, *IGKV1-5* was expressed by 7 and *IGKV1D-39* by 5 (Table II). However, frequent use of these *V*κ genes was not limited to IgG and therefore, it is unclear whether their use by anti-Dsg B cells is favored. IgM and IgG hybridomas differ in the frequency of *V*λ use (10 of 30 and 1 of 10, respectively) (Table II), but this difference was not significantly different (0.15; χ^2 test) owing to the small IgG sample size. Because V_L use is more diverse than V_H use among IgG hybridomas, hybridomas expressing the frequently used *IGV3-23* and *IGV3-30* were diverse in V_L gene use and included both *V*κ and *V*λ. Thus, restriction in IgG anti-Dsg structure does not extend to the level of H/L chain pairing, arguing that Dsg binding by IgG Abs is determined primarily by the H chain.

Somatic mutation of PV Abs

The sequence comparison to germline V_H and V_L genes reveals that anti-Dsg B cells have undergone somatic hypermutation. As expected, the IgG hybridomas are more extensively mutated than IgM hybridomas (9.4 vs 7.1 mutations per V_H, respectively). Moreover, approximately one-third of the IgM H chains were unmutated, and almost half had less than five mutations, whereas all IgG H chains were mutated and >90% have six or more mutations (Fig. 4). Analysis of the distribution of mutations suggests a bias in V_H mutation reflecting Ag selection. Mutations that result in amino acid replacement mutations in CDR-encoding regions of V_H or V_L are more likely to affect Ag binding than those in framework region (FWR)-encoding regions. Thus, Ag selection often results in an excess of R mutations in CDR-encoding regions, which is reflected by a high (more than two) amino acid replacement (R) to silent (S) ratio (R:S). Conversely, Ag selection often results in an absence of R mutations in FWR-encoding regions, which is reflected in a low R:S ratio (<2). In the absence of any selection, the R:S ratio would be ~2. The H chain gene somatic mutations for IgM hybridomas had a similar R:S ratio in FWR- (1.6) and CDR- (1.9) encoding regions (Table IV), whereas that for IgG H chain

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Table IV. *H and L chain replacement vs silent mutation (R/S) ratio of anti-Dsg Abs*

	H Chain (R/S)		L Chain (R/S)	
	FWR	CDR	FWR	CDR
IgM	79/41 (1.9)	66/41 (1.6)	38/13 (2.9)	31/18 (1.7)
IgG	70/61 (1.1)	86/17 (5.0)	34/35 (1.0)	40/17 (2.4)

genes was high in CDR- (5.0) and low in FWR- (1.1) encoding regions (Table IV). The R:S for L chain mutations of both IgM and IgG hybridomas was low in both CDR- and FWR-encoding regions with the exception of a slightly higher R:S in L chain FWR of IgM hybridomas (Table IV). Thus, the IgG hybridomas are more extensively mutated and exhibit a biased distribution of R somatic mutations to H chain CDRs, consistent with Ag selection of mutant IgG anti-Dsg B cells.

The presence of shared R mutations by clonally independent hybridomas is also suggestive of Ag selection of anti-Dsg B cells. Most of the shared mutations were located in H chain CDRs (Fig. 5). For example, among hybridomas expressing *IGHV3-23*, the CDR1 mutations S31N and S35T occurred twice, and the CDR2 mutations A50G, A50V, and G53A occurred twice, and the CDR2 mutation S57R occurred three times. Similarly, among hybridomas expressing *IGHV3-30*, the CDR1 mutations S31N and Y32F occurred four times and two times, respectively, while the CDR1 mutations G33A and G33T each occurred twice. Notably, the CDR1 mutation S31N occurred in both *IGHV3-23*- and *IGHV3-30*-expressing hybridomas, suggesting this mutation may be beneficial to Dsg binding by Abs encoded by either V_H gene. Shared R mutations were not limited to CDRs. For example, among *IGHV3-23*-expressing hybridomas, the FWR1 mutation L5V occurred three times and the FWR3 mutation K98R occurred twice (Fig. 5), and among *IGHV3-30*-expressing hybridomas, the FWR 1 mutation T28S occurred twice and the FWR3 mutation R98K occurred five times. Selection of FWR R mutations has been observed elsewhere, most notably among rheumatoid factor B cells (42). Altogether, there were 16 instances of the identical mutation occurring in two or more cells expressing the either *IGHV3-23* or *IGHV3-30* V_H gene, of which 11 occurred in CDRs and 5 in FWRs (Fig. 5). This occurrence of shared CDR R mutations provides further support for the idea that the IgG anti-Dsg B cells are Ag selected. Interestingly, some of these shared R mutations occurred in IgM hybridomas suggesting that mutant IgM anti-Dsg B cells have also been subject to selection for improved Ag binding.

The differences in somatic mutation among members of the same clone can be revealing about the nature of the mutations selected for by Ag. Only the large clonal set from patient PV5 was useful for this analysis because the members of all the other clonal sets were either identical in sequence or did not differ in R mutations. The hybridomas of patient PV5 had both shared and unique somatic mutations, indicating that somatic mutation was ongoing during clonal expansion (35). This pattern of somatic mutation allows the construction of a clonal tree based on the premise that shared mutations occurred before clonal divergence and unique mutations occurred after clonal divergence (Fig. 6). Interestingly, most of the early R mutations were nonconservative, while those that occurred later were generally conservative. Thus, five of the five R mutations shared by all seven hybridomas introduced a charge difference, whereas only two of the seven R mutations that occurred after divergence began involved a charge difference. This pattern is consistent with the notion that selection early in clonal selection was for mutant B cells with R mutations that improved Ag binding, whereas later in clonal expansion, selection was mainly for B cells with neutral mutations that did not disrupt Ag binding. This temporal pattern of mutation occurs in responses to both foreign and self-Ags (35, 43, 44). We are currently testing whether somatic mutation improves Dsg binding, as would be predicted if Dsg is the selecting Ag during clonal expansion.

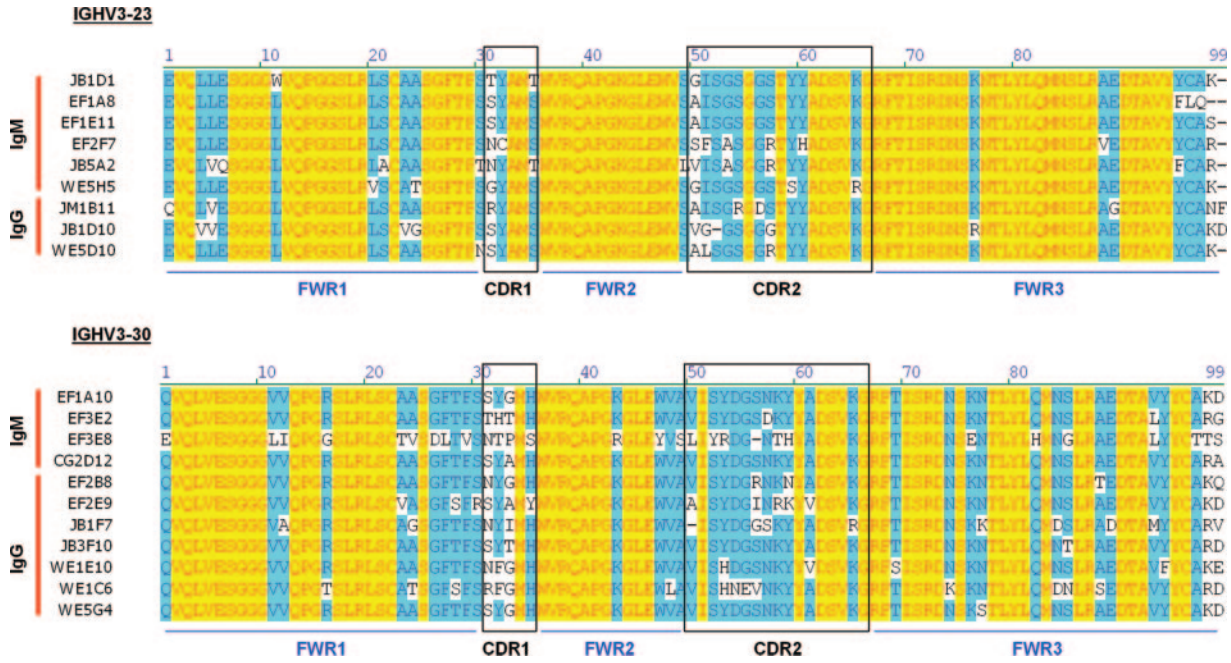

FIGURE 5. Hybridomas that expressed the *IGHV3-23* and *IGHV3-30* genes exhibited parallel mutations. The analysis was conducted using Vector NTI (Invitrogen Life Technologies). One member of a clone is shown, and thus, all mutations shown occurred independently. FWRs and CDRs are indicated and replacement mutations are highlighted. Positions in which no mutations occurred are in yellow, while positions in which mutations occurred are in blue with the specific mutation highlighted in white.

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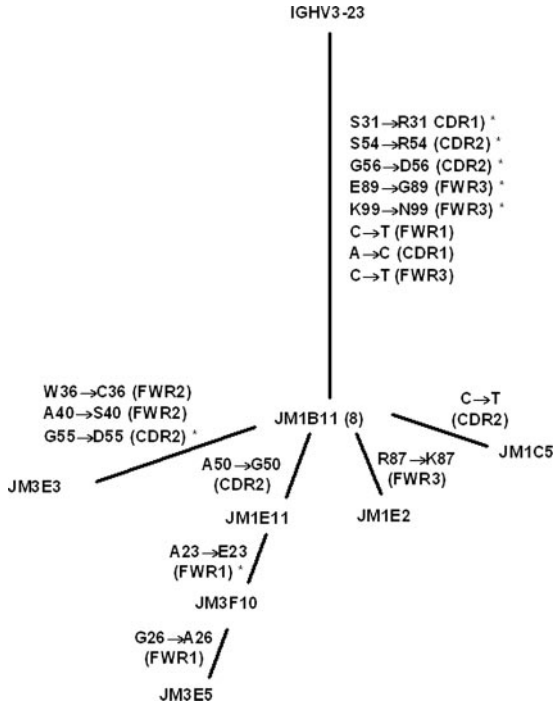

FIGURE 6. Phylogenetic analysis of the H chain genes from the large PV-5 clone indicates a temporal bias in somatic mutation. The tree was constructed based on the assumption that shared mutations occurred before clonal divergence and that unique mutations occurred after clonal divergence. The individual mutations and their location (CDR or FWR) are indicated along each branch. Replacement mutations are indicated by the amino acid substitution and silent mutations are indicated by the nucleotide substitution. *, Substitutions involving a change in charge. Each hybridoma is identified as in Table II. Eight hybridomas had the identical sequence and are represented by hybridoma JM1B11. The length of each line is proportional to the number of mutations between branch points.

Discussion

We have characterized a panel of 73 anti-Dsg hybridomas derived from six PV patients. Most of these hybridomas are IgM (64%) with all but one of the remainder IgG1. Our analysis reveals that mutant IgG anti-Dsg B cells, and to a lesser extent IgM anti-Dsg B cells, have been subject to ongoing Ag selection throughout Ag-driven clonal expansion potentially resulting in increased affinity for Dsg.

Several lines of evidence suggest Ag selection of IgG anti-Dsg B cells. First, the IgG hybridomas express a more restricted repertoire of V_H genes. Three-fourths of clonally independent IgG hybridomas express *IGHV3-30* or *IGHV3-23*. IgM anti-Dsg B cells also express these genes, but at a frequency that is not different from that in healthy individuals. This suggests that these two V_H genes provide an advantage for Dsg binding. Second, the IgG hybridomas are extensively mutated and the nature and distribution of these mutations are nonrandom. All IgG anti-Dsg hybridomas are mutated with nearly all having more than six V_H mutations, compared with IgM hybridomas in which approximately one-third are unmutated. V_H R mutations are biased for CDRs, particularly CDR2, and there are numerous examples of the same mutation occurring two or more times among clonally independent hybridomas. Moreover, analysis of the mutations among the large clone in patient PV-5 suggests a temporal bias in the nature of R mutations with nonconservative mutations generally occurring early in clonal expansion. We predict based on these findings that somatic mutation has increased the affinity of IgG Abs for Dsg. This suggests that Ag selection of IgG anti-Dsg B cells results in higher affinity mutants. Third, within individual patients the response is oligoclonal. Clonal sets of hybridomas were isolated from four of the six patients examined and they included two sets of IgG hybridomas. The most extreme example of oligoclonality is by patient PV-5, in which all 14 hybridomas are of a single expanded clone. Oligoclonality of a B cell response is a hallmark of secondary responses to foreign and self Ags and is attributed to a low precursor frequency of that clonotype in the preimmune repertoire and to the ability of somatic mutation to confer a growth advantage to just a few clones. Thus, altogether, these sequence data provide strong evidence of Ag selection of the anti-Dsg response in PV patients.

The IgM hybridomas also exhibit evidence of Ag selection. Many of these hybridomas are somatically mutated and, although there is no bias in the distribution of R mutations to CDR-encoding regions, there are examples of parallel V_H mutations between IgM and IgG hybridomas expressing *IGHV3-23* and *IGHV3-33*. In addition, there are several examples of clonally related IgM hybridomas in multiple patients suggesting that some IgM B cells have an advantage over others in Ag-driven clonal expansion. Two sets of clonally related IgM hybridomas were identified from patient PV-2, one of four hybridomas and the other of six, comprise over half of the hybridomas derived from this individual. Interestingly, the hybridomas of one of these clonal sets express *IGHV3-30* that is so commonly used by IgG anti-Dsg B cells. Caution in this interpretation is warranted, because many of the hybridomas of these clonal sets are not different in sequence, and therefore we cannot strictly rule out the possibility that they are an artifact of clonal expansion in vitro after EBV transformation. However, we note that all clonally related IgM hybridomas are somatically mutated, while half of the singlet IgM hybridomas are unmutated. This difference is significant (0.049; χ^2 test) suggesting that the clonal sets are a nonrandom group of IgM hybridomas, counter to the expectation if they were an artifact of the transformation process. Thus, these data suggest that IgM anti-Dsg B cells have undergone Ag selection resulting in clonal biases in some individuals.

It is noteworthy that approximately two-thirds of the anti-Dsg hybridomas were IgM. This too could be an artifact of the transformation process, as EBV transformation may preferentially immortalize IgM-expressing lymphocytes (45, 46). However, direct fusion of myeloma cells and PBLs from patients PV-4 and PV-6, although apparently less efficient, also yielded a majority (five of six) of IgM hybridomas, arguing against an artifact and suggesting that the predominance of IgM hybridomas reflects the repertoire of anti-Dsg B cells in the PB. Although a direct comparison between levels of IgM and IgG anti-Dsg Abs has not been made, serum IgG anti-Dsg is dramatically higher in patients than in controls, and thus the 2:1 IgM to IgG ratio reflected by the hybridoma analysis was higher than expected. This was especially so considering that the anti-Dsg response in these PV patients is longstanding and chronic, providing ample opportunity for IgG B cells to expand and dominate the repertoire, as occurs in secondary responses to foreign Ags (47). However, serum Abs should reflect the plasma cell repertoire and not necessarily the PB B cell repertoire, and thus, the anti-Dsg plasma cell repertoire may be predominantly IgG. Plasma cells, which reside in the tissues, especially the bone marrow, are rare in the blood and would not have been sampled in this study. Differences in the rate of IgM and IgG plasma cell differentiation could result in differences in isotype representation between blood B cells and tissue plasma cells. Alternatively, IgM and IgG plasma cells could have different half-lives (48). A short half-life for IgM plasma cells and a long half-life for IgG plasma

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cells would allow a minority of IgG plasma cells to contribute the majority of the serum anti-Dsg. Such a disparity in half-lives could result from parallel T-independent and T-dependent responses that generate short-lived IgM plasma cells and long-lived IgG plasma cells, respectively, as is suggested to occur (see below). Finally, the treatment regimen that these patients were undergoing at the time of blood draw could have selectively depleted the IgG B cells from circulation. Similar explanations may account for the unexpected absence of IgG4 anti-Dsg B cells, the pathogenic isotype of anti-Dsg Abs (49). Because all six patients have clinical disease, it is likely that they produce IgG4 anti-Dsg Abs, although the IgG4 anti-Dsg3 levels in sera from patients PV-1, PV-4, and PV-6 taken at the time of EBV transformation or fusion were not significantly different from healthy individuals (data not shown).

Most anti-Dsg autoantibodies (>90%) described here have dual specificity for Dsg1 and Dsg3. Dsg1 and Dsg3 share extensive homology in their ectodomains (EC1 to 5) (73% at EC1, 65% at EC2%, 57% at EC3, and 28% at EC4) (6), suggesting that these Abs bind a shared epitope. Because many IgM hybridomas are dual binders and unmutated, we conclude that dual binding B cells are present in the naive repertoire. Payne et al. (12) reported the characterization of monoclonal autoantibodies of dual specificity as well and determined that those with single specificity were not pathogenic. Dual binding Abs would account for the presence of both anti-Dsg1 and anti-Dsg3 Abs in mcPV patients (12). However, their presence among mPV patients is at odds with the fact that the majority of patients with this form of the disease have only anti-Dsg3 antibodies (4, 5). Thus, these data indicate an additional difference between the anti-Dsg B cell repertoire in PB and the serum anti-Dsg Abs. This difference may be related to which anti-Dsg B cells become long-lived plasma cells, as discussed above.

Recently, we have identified IgM and IgG anti-Dsg1 autoantibodies in the sera of the endemic form of PF, known as fogo selvagem (FS) in Brazil (L. Diaz, P. Prisayanh, D. Dasher, N. Li, F. Evangelista, V. Aoki, G. Hans-Filho, V. Santos, B. Qaqish, and E. Rivitti, submitted for publication). These IgM anti-Dsg1 autoantibodies are distinctive of FS patients and normal individuals living in rural endemic areas of the disease. Pathogenic IgG anti-Dsg1 autoantibodies are present only in patients (25, 49). However, there are nonpathogenic IgG anti-Dsg1 and anti-Dsg3 autoantibodies in the sera of normal individuals and patients during the preclinical stages of FS (25, 26). It is proposed that IgM anti-Dsg1 autoantibodies may be in response to an environmental Ag(s) by individuals of these endemic regions. This IgM response may be followed by production of nonpathogenic IgG anti-Dsg1 in certain individuals, and in a few genetically predisposed individuals, to the generation of pathogenic IgG and FS disease. Our analysis of PV patients showing that IgM anti-Dsg B cells are in some cases extensively mutated (Table II) with some showing evidence of having undergone Ag selection (Fig. 5), albeit less stringently than IgG anti-Dsg B cells, is consistent with this hypothesis. What is responsible for the production of IgM anti-Dsg Abs, which are nonpathogenic and perhaps protective, and for the conversion to the production of mutant and Ag-selected IgG anti-Dsg Abs, particularly the pathogenic IgG4 Abs, will be important to understanding the etiology of these skin diseases and for the rational design of new treatment modalities.

Disclosures

The authors have no financial conflict of interest.

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