



Genetic characterization of human Dsg3-specific B cells isolated by flow cytometry from the peripheral blood of patients with pemphigus vulgaris

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KEYWORDS

Antigen-specific;
Autoimmune disease;
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Summary

Background: Pemphigus vulgaris (PV) is an autoimmune bullous disease caused by anti-desmoglein 3 (Dsg3) IgG autoantibodies; however, the Dsg3-specific B cells that produce anti-Dsg3 IgG are not well characterized.

Objectives: Our aims were to develop a flow cytometric method for the isolation of Dsg3-specific B cells from the peripheral blood of patients with active PV and to identify the variable regions within their heavy- and light-chain immunoglobulin genes.

Methods: Dsg3-specific B cells were isolated as CD3⁺IgD⁺PI⁺Dsg3E-tag⁺ cells using recombinant human Dsg3 with an E-tag (rDsg3-E-His) as a probe. Heavy- and light-chain cDNA was produced by PCR from single B cells; these were used to characterize the usage and CDR3 sequence in the variable region of each gene.

Results: Staining conditions were optimized using mouse hybridoma cells against human Dsg3 and peripheral blood mononuclear cells (PBMCs) from a PV patient. Individual Dsg3-specific B cells were isolated by FACS from four PV patients at a frequency of 1–18 per 10⁵ PBMCs. CDR3 sequences and identical gene usage in the variable region were identified in several B cells from the same PV patients. Common gene usage was also found among several PV patients.

Conclusions: These results suggested clonal expansion of autoreactive B cells and restricted gene usage for autoreactive B cells against Dsg3. Our method for the isolation of Dsg3-specific B cells will allow the systematic analysis of immunoglobulin gene usage in PV patients, which may elucidate the mechanism of immunopathogenesis.

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Abbreviations: PV, pemphigus vulgaris; PBMCs, peripheral blood mononuclear cells; Dsg1, desmoglein 1; Dsg3, desmoglein 3; Ig, immunoglobulin.

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1. Introduction

Pemphigus vulgaris (PV) is a tissue-specific autoimmune disease mediated by IgG autoantibodies that bind the desmogleins (Dsg) found in desmosomes. Dsg is a cadherin-type cell–cell adhesion molecule that plays a key role in mediating the adhesion between keratinocytes [1]. Dsg3 is the primary autoimmune target of PV, and essentially all patients with PV have anti-Dsg3 IgG autoantibodies [2,3]. These antibodies have direct pathogenic effects, including the loss of cell–cell adhesion in the epidermis and mucous membranes, with resultant acantholytic blisters [4,5].

Although the immunological mechanisms underlying the production of autoantibodies in pemphigus remain to be clarified, it was previously demonstrated that susceptibility to PV is associated with certain major histocompatibility complex (MHC) class II alleles, including DRB1*0402, DRB1*1401, and DQB1*0302 in Caucasians and DRB1*14 and DQB1*0503 in Japanese individuals [6–9]. This susceptibility suggests that MHC class II-restricted CD4⁺ T cells are involved in the production of pathogenic IgG autoantibodies in PV. In fact, some Dsg3-specific T cell lines or clones were isolated using recombinant proteins or peptides representing the various extracellular domains of Dsg3 [10,11].

Analyses of the antigen-specific B cells in autoimmune bullous diseases have been hampered by technical difficulties in isolating or culturing B cells from patients; however, several recent studies have successfully found ways to analyze the antigen-specific B cells involved in autoimmune bullous diseases. Dsg3-specific B cells were detected in the peripheral blood of patients with active PV using enzyme-linked immunospot assay [12], and memory B cells specific for the 180-kDa bullous pemphigoid antigen were enriched from the peripheral blood of patients with bullous pemphigoid [13]. In addition, modern techniques using phage display [14,15] and a mouse–human heterohybridoma [16] have allowed us to genetically characterize antigen-specific B cells from PV patients.

Our aim was to develop a flow cytometric method for the isolation of individual Dsg3-specific B cells from the peripheral blood of patients with active PV and to analyze the gene usage and sequences of the variable (V) regions in the genes encoding their Dsg3-specific IgG autoantibodies using PCR. This is the first research to isolate individual Dsg3-specific B cells from the peripheral blood of PV patients.

2. Materials and methods

2.1. Blood samples

Peripheral blood mononuclear cells (PBMCs) were obtained from four patients (two male and two female) with PV that were diagnosed on the basis of the following clinical and laboratory findings: (1) typical clinical features with blisters and erosions in the oral mucosa, (2) suprabasal acantholysis detected by histological examination, (3) *in vivo* surface deposition of IgG on the keratinocytes detected using direct immunofluorescence, and (4) circulating anti-Dsg3 IgG autoantibodies detected using ELISA. Three of them are active mucocutaneous PV without treatment and one of them is mucosal-dominant PV, who has had disease for 15 years, treated with 10 mg/day prednisolone. The patients' clinical information is organized in Table 1. This study was approved by the Keio University Institutional Review Board. Written informed consent was obtained from all subjects.

2.2. Production of recombinant proteins

The recombinant proteins used, i.e., rDsg3-E-His and rDsg1-E-His, were produced by a baculovirus expression system as described previously [17]. High Five cells (Invitrogen, San Diego, CA, USA) cultured in serum-free EX medium (JRH Bioscience, Lenexa, KS, USA) were infected with recombinant virus and incubated at 27 °C for 3–4 days. The recombinant proteins produced in the culture supernatants were purified on TALON affinity metal resin (Clontech,

Table 1 Clinical information and frequency of Dsg3-specific B cells for each patient

	Age	Sex	Diagnosis	Oral lesion	Skin lesion	Dsg1	Dsg3	Duration of the disorder	Treatment	Frequency of Dsg3-specific B cells
Patient 1	56	M	MCPV	+	+	54.1	209.7	2 months	No treatment	18/1 × 10 ⁵ PBMC
Patient 2	49	F	MCPV	+	+	36.6	221.5	9 months	No treatment	3/1 × 10 ⁵ PBMC
Patient 3	35	F	MCPV	+	+	109.9	181.8	7 months	No treatment	1/1 × 10 ⁵ PBMC
Patient 4	53	M	MDPV	+	–	3.5	234	15 years	PSL 10 mg/day	8/1 × 10 ⁵ PBMC

MCPV, mucocutaneous PV; MDPV, mucosal-dominant PV; Dsg1, Dsg1 ELISA index; Dsg3, Dsg3 ELISA index; PSL, prednisolone.

Palo Alto, CA, USA) according to the manufacturer's recommendations.

2.3. Isolation of single Dsg3-specific B cells from the PBMCs of PV patients

We developed a strategy for the isolation of individual B cells specific for Dsg3 from the PBMCs of patients with PV by flow cytometry (Fig. 1). PBMCs from PV patients were first incubated with recombinant Dsg3 (Dsg3-E-His) expressed by a baculovirus system with an E-tag for detection and a His-tag for purification at the C-terminal tail, which is known to have the proper three-dimensional structure with conformational epitopes [4,17]. Dsg3-specific B cells express Dsg3-specific Ig receptors on their surfaces; therefore, they should be recognized by Dsg3-E-His and anti-E-tag antibodies conjugated with Alexa488. The negative fractions were excluded using an anti-CD3 antibody that binds T cells, an anti-IgD antibody that binds B cells lacking IgG expression (i.e., cells that express both IgD and IgM on their surface have no IgG class-switching) [18], and propidium iodide (PI) to reduce background staining. Accordingly, the Dsg3-specific IgG-expressing B cells were detected as a CD3⁻IgD⁻PI⁻E-tag⁺ population. We thus attempted to isolate Dsg3-specific cells by a single-sorting procedure using flow cytometry, not in

combination with sorting using magnetic beads or other procedures.

2.4. Flow cytometric analysis with hybridoma cells

The AK9 and AK23 hybridoma cells used have been described previously [19]. Although the AK series of mAbs were isolated as antibodies that bind mouse Dsg3, some AK mAbs also bind human Dsg3. The AK23 mAb, but not the AK9 mAb, binds human Dsg3. A total of 1×10^6 AK9 or AK23 hybridoma cells were incubated with 10 μ g of rDsg3-E-His or rDsg1-E-His in 100 μ l of RPMI medium on ice for 1 h, then incubated with 1 μ l of Alexa488-conjugated anti-E-tag mouse monoclonal antibody (mAb; Amersham Biosciences, Buckinghamshire, UK) for 30 min at room temperature, and analyzed using a FACSCalibur[®] flow cytometer (Becton Dickinson, San Diego, CA, USA). The Alexa488-conjugated anti-E-tag mouse mAb was made using an Alexa488 mAb labeling kit (Invitrogen).

2.5. Flow cytometric analysis with PBMCs

PBMCs from four PV patients and four healthy control subjects were isolated by density gradient centrifugation on Lymphoprep[®] (Nycomed Pharma AS, Oslo, Norway). A total of 5×10^6 PBMCs were incu-

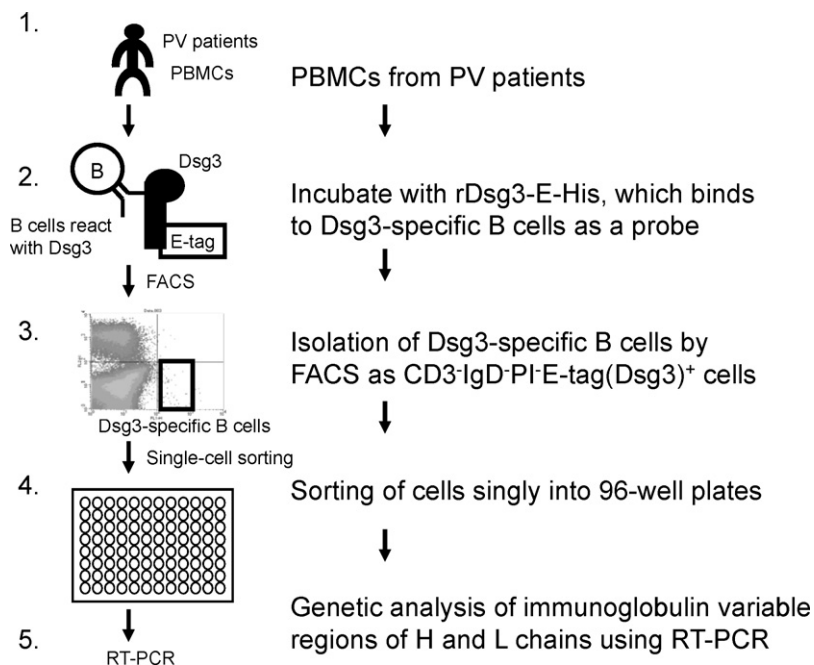


Fig. 1 The sorting of single Dsg3-specific B cells from the PBMCs of patients with PV. PBMCs from PV patients were incubated with rDsg3-E-His as a specific probe, and Dsg3-specific B cells were sorted as CD3⁻IgD⁻PI⁻E-tag⁺ cells by FACS. B cells were sorted singly into wells of a 96-well plate. The cDNA corresponding to the immunoglobulin variable regions was amplified by RT-PCR, and the sequences were analyzed.

bated with 10 μ g of recombinant rDsg3-E-His or rDsg1-E-His in 100 μ l of RPMI medium on ice for 1 h. After washing with RPMI, the cells were incubated with 5 μ l of FcR blocking reagent (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 min at room temperature and then with 1 μ l of Alexa488-conjugated anti-E-tag antibody, 5 μ l of phycoerythrin (PE)-conjugated anti-IgD antibody (Becton Dickinson), and 5 μ l of PE-Cyanin 5 (PE-Cy5)-conjugated anti-CD3 antibody (Becton Dickinson) for 30 min at room temperature. After incubation with 15 μ l of propidium iodide (PI) for 15 min before analysis, the specimens were analyzed using a FACS-Calibur[®] flow cytometer (Becton Dickinson). We used PBMCs from patient 1 for staining comparison with Dsg1 and Dsg3. Even though the patient 1 had both anti-Dsg1 and anti-Dsg3 antibodies, the titer of Dsg1 was much lower than that of Dsg3, and therefore, we assumed that Dsg1 could be used as a negative staining control antigen.

2.6. Isolation of Dsg3-specific B cells by single-cell sorting

A total of 2.5×10^7 PBMCs were incubated with 50 μ g of rDsg3-E-His in 500 μ l of RPMI medium on ice for 1 h. After washing with RPMI, the cells were incubated with 5 μ l of FcR blocking reagent for 5 min at room temperature and then with 1 μ l of Alexa488-conjugated anti-E-tag, 5 μ l of PE-anti-IgD, and 5 μ l of PE-Cy5-anti-CD3 for 30 min at room temperature. After incubation with 15 μ l of PI for 15 min, the cells were sorted using an EPICS[®] ALTRA (Beckman Coulter, Fullerton, CA, USA). All analyses were performed on the lymphocyte fraction, and gating was based on the forward and side scatters. We isolated the Dsg3-specific B cells as a CD3⁺IgD⁺PI⁺E-tag⁺ population. The sorted cells were then individually deposited into the wells of a 96-well plate (MJ Research, Waltham, MA, USA). Because each FACS machine had a different scale on its axes, the scales between which the Dsg3-specific B cells were sorted changed from the previous flow cytometric analyses (FACS-Calibur[®] flow cytometer, Becton Dickinson) (see Section 2.5).

2.7. RT-PCR and sequence analysis of the immunoglobulin (Ig) genes

For RT-PCR, the B cells were sorted into wells containing 5 μ l of Cells to Signal[®] lysis buffer (Ambion, Austin, TX, USA), and total RNA was isolated from each cell. First-strand cDNA was prepared from the RNA samples using Transcriptor (Roche, Basel, Switzerland) in the presence of random hexamer primers according to the manufacturer's protocol. Three

microliters of each cDNA solution were used to amplify the IgH, Ig λ , and Ig κ transcripts by two rounds of PCR in 20 μ l reactions containing 0.5 μ M primers and 1 U of HotstarTaq Master Mix (Qiagen, Hilden, Germany). Each round of PCR was performed for 45 cycles (first PCR) or 30 cycles (second PCR) at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s. The primers used for each round of PCR (first PCR, including the Ig constant region; second PCR, Ig variable region-specific) are described elsewhere [20]. The amplified products were fractionated on agarose gels and visualized by ethidium bromide staining. All products were purified using a PCR clean-up kit (Promega, Madison, WI, USA) and then sequenced and analyzed using Ig BLAST (<http://www.ncbi.nlm.nih.gov/igblast/>) or the International Immunogenetics Information System (<http://imgt.cines.fr/>) and compared with the GenBank database.

3. Results

3.1. Specific binding of Dsg3-E-His to Dsg3-specific mouse hybridoma cells

We first optimized the various parameters of our staining conditions with Dsg3-E-His using mouse hybridoma cells, which produce anti-Dsg3 AK mAbs [19]. Although the AK series of mAbs were isolated as antibodies that bind mouse Dsg3, some AK mAbs also bind human Dsg3. The AK23 mAb, but not the AK9 mAb, binds human Dsg3. We used AK23 hybridoma cells as a positive control and AK9 hybridoma cells as a negative control. When AK23 or AK9 hybridoma cells were incubated with human Dsg3-E-His, human Dsg1-E-His, or without recombinant protein, the AK23 hybridoma cells were clearly detected with Dsg3-E-His, but not with Dsg1-E-His (Fig. 2). The AK9 hybridoma cells were recognized as negatively stained cells with Dsg3-E-His. These findings demonstrate the feasibility of our staining protocol.

3.2. Detection of Dsg3-specific B cells among the PBMCs of a PV patient using flow cytometry

We next determined whether Dsg3-specific B cells could be detected among the PBMCs of patients with PV using the above staining conditions. We incubated PBMCs from patients with PV or normal individuals with Dsg3-E-His or Dsg1-E-His on ice to prevent internalization, then with a cocktail containing anti-E-tag, anti-CD3, and anti-IgD antibodies. When 5×10^5 PBMCs from a PV patient were incubated with Dsg3-E-His or Dsg1-E-His, 87

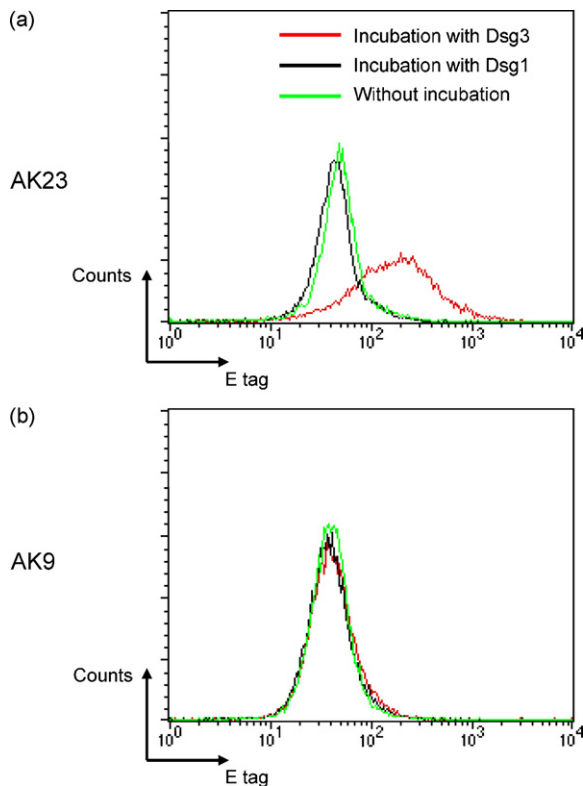


Fig. 2 Detection of Dsg3-specific hybridoma cells with rDsg3-E-His using flow cytometry. (a) AK23 hybridoma cells that reacted with human Dsg3 were stained with rDsg3-E-His, but not rDsg1-E-His. (b) AK9 hybridoma cells that did not react with human Dsg3 were not stained with rDsg3-E-His or rDsg1-E-His.

CD3⁺IgD⁺PI⁺E-tag⁺ cells were detected with Dsg3-E-His, whereas only 18 CD3⁺IgD⁺PI⁺E-tag⁺ cells were detected with Dsg1-E-His. In addition, when 5×10^5 PBMCs from four healthy control were incubated with Dsg3-E-His, only 20, 11, 21, and 11 CD3⁺IgD⁺PI⁺E-tag⁺ cells were detected, respectively (Fig. 3). Dsg3-specific B cells were detected in the area for which the E-tag signal was medium-positive, which is consistent with our results using AK23 hybridoma cells. These results indicate that Dsg3-specific B cells can be detected as CD3⁺IgD⁺PI⁺E-tag⁺ cells from the PBMCs of PV patients using our staining protocol, although a quarter of positive cells could be due to non-specific staining.

3.3. Sorting of Dsg3-specific B cells from the PBMCs of patients with PV

We next used our staining procedure to sort Dsg3-specific B cells from the PBMCs of four PV patients and one healthy control subject. In our sorting protocol, after gating for the lymphocyte population based on the forward and side scatters, an IgD⁺

and E-tag⁺ population was separated from the CD3-negative population as Dsg3-specific B cells (representative data in Fig. 4). The sorted cells were then deposited individually into a 96-well plate until all of the wells were filled. CD3⁺IgD⁺PI⁺E-tag⁺ cells were sorted at a frequency of 1–18 cells per 1.0×10^5 PBMCs (Table 1). In contrast, no cells were sorted from 1×10^5 PBMCs from the healthy control subject (Fig. 4).

3.4. Amplification of the Ig V regions in the sorted B cells by RT-PCR

Individual cells were sorted on a 96-well plate filled with lysis buffer, total RNA was isolated, and cDNA was synthesized. An initial round of PCR was performed using primers specific for the V and constant regions of each Ig chain (i.e., the heavy chain and κ and λ light chains). A second round of amplification using Ig V-region-specific primers was attempted for each sample that was positive in the first round. The cDNA was theoretically synthesized from individual Dsg3-specific B cells, but it was technically difficult to obtain PCR products from all of the sorted cells. For example, in patient 1, the number of positive bands after the first round of PCR was 28 for the heavy chain, 30 for the λ chain, and two for the κ chain out of 96 sorted cells; however, many of the cells showed either a heavy- or light-chain product, and 13 of the samples that had a pair of heavy- and light-chain products in the first round of PCR had two pairs of heavy- and light-chain products (representative data in Fig. 5). Among the four PV patients studied, 19, 16, and 17 products were obtained for the V regions of the heavy, κ , and λ light chains, respectively, whereas 11 complete sets of heavy- and light-chain products were obtained (Table 2).

3.5. Sequencing of the Ig V regions

We determined the sequences of the V regions in the heavy and light chains from our single-sorted Dsg3-specific B cells and compared the third complementarity-determining region (CDR3) and gene usage of the V region from each sorted cell with that in the human genome database (Table 2). In patient 1, two pairs of heavy- (VH) and light-chain (VL) sequences were obtained in addition to two VH sequences and five VL sequences without their counterparts. In patient 2, seven pairs of VH and VL sequences were obtained in addition to four solitary VH sequences and 10 solitary VL sequences. In patient 3, a pair of VH and VL sequences, one solitary VH sequence, and three solitary VL sequences were collected. In patient 4, a pair of VH and VL sequences, one

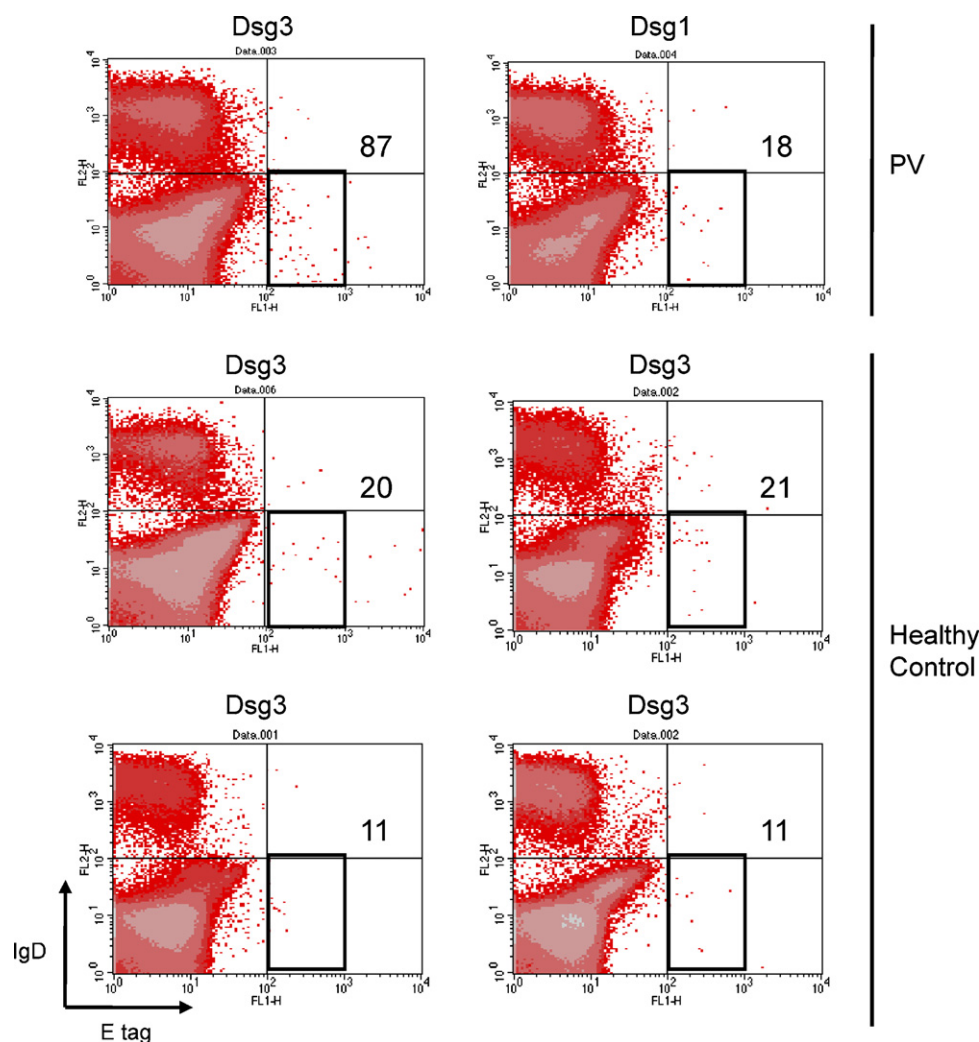


Fig. 3 Detection of Dsg3-specific B cells from the PBMCs of a patient with PV. Using 5×10^5 PBMCs from a PV patient, Dsg3-specific B cells were detected as CD3[−]IgD[−]PI[−]E-tag⁺ cells with rDsg3-E-His, although some background staining occurred as determined incubating PBMCs from the PV patient with rDsg1-E-His or PBMCs from healthy control subjects with rDsg3-E-His.

solitary VH sequence, and four solitary VL sequences were obtained.

In general, for the patients with PV ($n = 4$), VH1, VH3, and VH4 were predominantly used in the single-sorted B cells (Table 2). Identical gene usage in the V regions was observed in different single-sorted B cells from the same PV patient. Three sorted B cells with V2-14 and two sorted B cells with V1-3 in the VL gene were isolated from patient 1. In comparison, five sorted B cells with VH1-46, two sorted B cells with VH3-15, and two sorted B cells with VH3-73 in the VH gene were isolated from patient 2. Notably, three sorted B cells with V2-17, two sorted B cells with V1-4, three sorted B cells with A18b, and three sorted B cells with A23 in the VL gene were obtained from the same patient. In addition, two sorted B cells with V1-13 were isolated from patient 3. Further-

more, a number of sorted B cells had identical CDR3 sequences in both *VH* and *VL*, as found in patient 2 (wells 1E and 6A). Other cells had identical CDR3 sequences in either of each chain, as found in patient 2 (wells 10D and 10G; AGGDWSSRSFDY) and patient 3 (wells 4D and 11A; QSSDSSRTYR). More interestingly, several common gene usages were noticed among different PV patients, including VH3-73 in patients 2 and 3, VH4-59 in patients 1 and 4, V1-22 in patients 1 and 3, V1-16 in patients 2 and 3, and V1-4 in patients 2 and 4. Furthermore, one CDR3 λ chain sequence (QSSDSSRTYR) was identical in the sorted B cells from patients 2 and 3. These findings suggest that the Dsg3-specific B cells in PV patients proliferate by clonal expansion and that PV patients may have restricted gene usage in the V regions of their Dsg3-specific IgG autoantibodies.

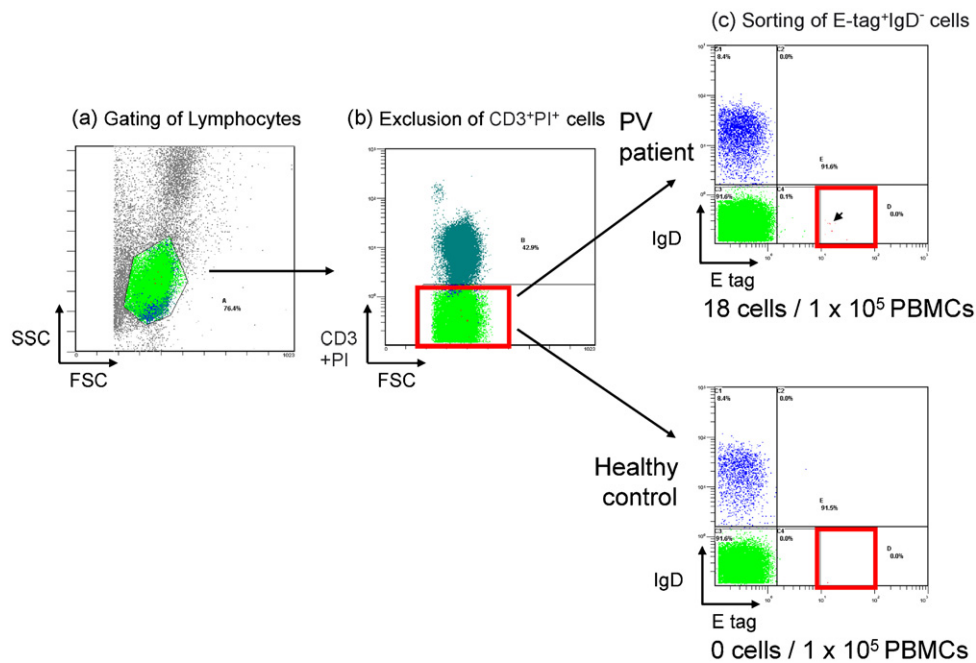


Fig. 4 Sorting of Dsg3-specific B cells from the PBMCs of PV patients. Lymphocytes were extracted by gating based on the forward and side scatter screens during flow cytometry from the PBMCs of patients with active PV. (a) After exclusion of the PI^+CD3^+ population ((b) $CD3^+$: dark green, $CD3^-$: light green), an IgD^- and $E\text{-tag}^+$ population was sorted as Dsg3-specific mature B cells ((c) arrowhead). No event in this population was detected in the PBMCs from a healthy control subject. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

4. Discussion

To characterize the Ig gene for the Dsg3-specific IgG autoantibodies in PV patients, we overcame two technical hurdles. First, it was necessary to isolate individual Dsg3-specific B cells from the PBMCs of patients with PV using flow cytometry. We optimized our staining conditions and succeeded in obtaining single B cells using rDsg3-E-His, which binds to the Ig receptors on Dsg3-specific B cells, and we subsequently incubated them with fluorescent-labeled anti-E-tag antibodies. Second, we performed sequence analysis of single B cells in 96-well plates using RT-PCR. The cDNA was synthesized from each B cell, which was then subjected to two-step PCR: the first round of PCR amplified the constant and V regions, whereas the second round of PCR was specific for the V region.

Several of the Dsg3-specific B cells that were isolated from the same patient had identical gene usage in the V region and identical CDR3 sequences. This suggests that Dsg3-specific autoreactive B cells proliferate by clonal expansion in PV patients. Moreover, we found that one λ CDR3 sequence was identical among the B cells isolated from different patients, suggesting the possible involvement of this sequence in Dsg3-binding. VH1, VH3, and VH4 were frequently detected. It is unclear whether this restricted usage has any significance for anti-Dsg3 IgG autoantibody production in pemphigus or whether it reflects the gene usage in the majority of IgGs [21,22]. Additional studies with a large number of patients from various ethnic groups are necessary to clarify this issue.

The frequency of Dsg3-specific B cells in the PBMCs was calculated as 1–18 per 10^5 cells based

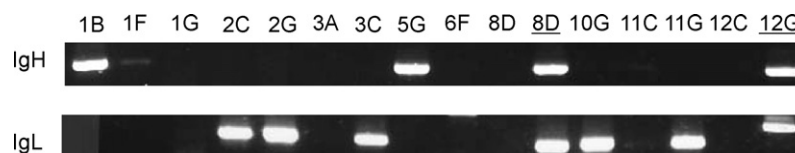


Fig. 5 Amplification of the immunoglobulin variable region using RT-PCR. Positive bands for the heavy and light chains from the sorted cells are shown as representative data for patient 1. Each lane represents one single sorted cell. The cells in wells 8D and 12G had amplified bands for a pair of heavy and light chains (underlined).

Table 2 Gene usage of the immunoglobulin variable region and sequence of the CDR3 region in sorted B cells from PV patients

	VH					VL			
	Position	V-gene	J-gene	CDR3		CDR3 length	V-gene	J-gene	CDR3
Patient 1	8D	VH5-51	JH5	ARHGPNWGFRC	12	V2-14	JL2	QVWDSNGDH	9
	12G	VH4-34	JH6	ATGFGRLMSYY	11	L14	JK1	LQHNNYP	7
	1B	VH4-59	JH5	AKEGPGYSSGSFEI	14	ND			
	5G	VH1-18	JH2	ARDPAWHPKLRPP	13	ND			
	2C	ND				V2-14	JL1	QVWDSHTAQ	9
	3G	ND				V2-14	JL1	EVWDSNDH	9
	2G	ND				V1-3	JL3	CSYADNYTL	9
	11G	ND				V1-3	JL1	CSYGGSYSLGI	11
	10G	ND				V1-22	JL3	QSYDSQNL	8
Patient 2	1E	VH3-73	JH6	TRRDVSAHRSDSDMDV	16	V2-17	JL3	QSSDSSRTYR	10
	6A	VH3-73	JH6	TRRDVSAHRSDSDMDV	16	V2-17	JL3	QSSDSSRTYR	10
	5B	VH3-15	JH5	TTFLGAEVATEW	12	A18b	JK2	MQGVHPL	7
	6B	VH3-15	JH5	TTFLGAEAGTEWFDP	15	A18b	JK2	MQSIHPLY	8
	8C	VH3-49	JH5	TRDGEYQLRMAGGGRPRW	18	L2	JK1	QQYNNWPR	8
	4F	VH1-46	JH3	ARDRSGIAARPHAS	14	A2	JK4	MQSVQLP	7
	3H	VH1-46	JH3	ASGRAFDI	8	A1	JK3	MQATHWPPI	9
	10D	VH1-46	JH4	AGGDWSSRSFDY	12	ND			
	10G	VH1-46	JH4	AGGDWSSRSFDY	12	ND			
	7B	VH1-46	JH3	ARDRSGVPARPHAF	14	ND			
	12C	VH3-30	JH5	AKSGEVASSYGSVSYLNL	18	ND			
	2A	ND				A23	JK2	MQATDFPY	8
	6C	ND				A23	JK2	TQTIQFP	7
	7D	ND				A23	JK2	TQATHFP	7
	1B	ND				V1-4	JL1	SSYTTNSL	8
	8D	ND				V1-4	JL3	SSYTSISTW	9
	9F	ND				V2-17	JL3	QSSDSSRTYR	10
	9B	ND				A18b	JK4	MQSVQFP	7
	4D	ND				V1-16	JL3	AAWDDSLRGP	10
	8F	ND				A27	JK4	QQYGGSPA	8
	10H	ND				L6	JK5	QQRIKWPL	8
Patient 3	1D	VH3-73	JH4	ARDRTGLKARPHG	13	V1-22	JL3	CSYAGSSTFR	10
	3C	VH3-74	JH6	ADFGRNGLA	9	ND			
	4D	ND				V1-13	JL3	QSSDSSRTYR	10
	10H	ND				V1-13	JL3	QSYDSVLIGW	10
	11A	ND				V1-16	JL3	QSSDSSRTYR	10
Patient 4	9C	VH1-3	JH5	GVNYYGSGRDWLDP	14	B3	JK4	QQYYNTP	7
	5B	VH4-59	JH4	QDYDSGSNYNGGWFP	16	ND			
	1B	ND				V1-4	JL3	SSYASTSTI	9
	7A	ND				A27	JK2	QQYGVSP	8
	7H	ND				A19	JK5	MQGLQNPL	8
	10D	ND				B3	JK1	QQYYSSR	7

ND, not detected.

on the number of sorted B cells in our assay. The frequency of antigen-specific B cells (anti-glycoprotein IIb/IIIa antibody-producing B cells) in patients with immune thrombocytopenic purpura was reported to be 2–30 per 10⁵ PBMCs [23], whereas the frequency of Hepatitis B surface antigen (HBsAg)-specific B cells was determined to be 54.9 per 10⁵ PBMCs after vaccination using enzyme-linked immunospot (ELISPOT) assay [24]. These figures provide an idea of the frequency of antigen-specific B cells in circulation because they fall within a similar range.

The characteristics of the Ig V regions in pemphigus patients have been investigated previously using

different approaches [14,16]. One of the most recent advances is phage display, which has been used to obtain single-chain V-region fragments (scFvs) and several human mAbs against Dsg3 and Dsg1 from a PV patient [14]. In this system, a phage library is constructed from the PBMCs of a patient; the library contains Dsg3-specific B cells, as well as a large population of other non-specific B cells. The phage clones that bind to Dsg3 are then selected and expanded by panning from this library. Because the pairs of the V regions for the heavy and light chains are randomly shuffled during the construction of the scFv library, the pairs of isolated scFvs may not reflect the heavy- and light-chain pairings *in vivo*

[14]. Our approach is advantageous in that it eliminates the possibility of mispairing because the isolation of the heavy and light chains is done directly on each sorted B cell.

However, the weakness of this study is that we could not directly prove that the isolated heavy- and light-chain pairs actually reacted with Dsg3 by constructing scFv from the isolated pairs. The reason for this failure partly includes technical reasons such as insolubility of some of scFv tested. On the other hand, the isolation of antigen-specific B cells from patients' PBMCs will allow us to perform not only the sequence analyses of heavy- and light-chain variable regions but also microarray analysis for gene expression profiling such as cytokines or surface markers in the future studies.

In summary, we have developed a novel strategy for the isolation of Dsg3-specific B cells from the peripheral blood of PV patients. We also analyzed the gene usage and sequences of the V regions in the genes encoding their IgG autoantibodies. Our approach will be a valuable tool for future characterization of the B cell repertoire in pemphigus and it may be used to elucidate the mechanism of pathogenesis.

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References

- [1] Green KJ, Gaudry CA. Are desmosomes more than tethers for intermediate filaments? *Nat Rev Mol Cell Biol* 2000;1:208–16.
- [2] Amagai M, Klaus-Kovtun V, Stanley JR. Autoantibodies against a novel epithelial cadherin in pemphigus vulgaris, a disease of cell adhesion. *Cell* 1991;67:869–77.
- [3] Stanley JR, Amagai M. Pemphigus, bullous impetigo, and the staphylococcal scalded-skin syndrome. *N Engl J Med* 2006;355:1800–10.
- [4] Amagai M, Hashimoto T, Shimizu N, Nishikawa T. Absorption of pathogenic autoantibodies by the extracellular domain of pemphigus vulgaris antigen (Dsg3) produced by baculovirus. *J Clin Invest* 1994;94:59–67.
- [5] Amagai M, Ahmed AR, Kitajima Y, Bystryk JC, Milner Y, Gniadecki R, et al. Are desmoglein autoantibodies essential for the immunopathogenesis of pemphigus vulgaris, or just “witnesses of disease”? *Exp Dermatol* 2006;15:815–31.
- [6] Ahmed AR, Wagner R, Khatri K, Notani G, Awdeh Z, Alper CA, et al. Major histocompatibility complex haplotypes and class II genes in non-Jewish patients with pemphigus vulgaris. *Proc Natl Acad Sci USA* 1991;88:5056–60.
- [7] Ahmed AR, Yunis EJ, Khatri K, Wagner R, Notani G, Awdeh Z, et al. Major histocompatibility complex haplotype studies in Ashkenazi Jewish patients with pemphigus vulgaris. *Proc Natl Acad Sci USA* 1990;87:7658–62.
- [8] Miyagawa S, Higashimine I, Iida T, Yamashina Y, Fukumoto T, Shirai T. HLA-DRB1*04 and DRB1*14 alleles are associated with susceptibility to pemphigus among Japanese. *J Invest Dermatol* 1997;109:615–8.
- [9] Niizeki H, Inoko H, Mizuki N, Inamoto N, Watababe K, Hashimoto T, et al. HLA-DQA1, -DQB1 and -DRB1 genotyping in Japanese pemphigus vulgaris patients by the PCR-RFLP method. *Tissue Antigens* 1994;44:248–51.
- [10] Hertl M, Amagai M, Sundaram H, Stanley J, Ishii K, Katz SI. Recognition of desmoglein 3 by autoreactive T cells in pemphigus vulgaris patients and normals. *J Invest Dermatol* 1998;110:62–6.
- [11] Veldman CM, Gebhard KL, Uter W, Wassmuth R, Grotzinger J, Schultz E, et al. T cell recognition of desmoglein 3 peptides in patients with pemphigus vulgaris and healthy individuals. *J Immunol* 2004;172:3883–92.
- [12] Nishifuji K, Amagai M, Kuwana M, Iwasaki T, Nishikawa T. Detection of antigen-specific B cells in patients with pemphigus vulgaris by enzyme-linked immunospot assay: requirement of T cell collaboration for autoantibody production. *J Invest Dermatol* 2000;114:88–94.
- [13] Leyendeckers H, Tasanen K, Bruckner-Tuderman L, Zillikens D, Sitaru C, Schmitz J, et al. Memory B cells specific for the NC16A domain of the 180 kDa bullous pemphigoid autoantigen can be detected in peripheral blood of bullous pemphigoid patients and induced in vitro to synthesize autoantibodies. *J Invest Dermatol* 2003;120:372–8.
- [14] Payne AS, Ishii K, Kacir S, Lin C, Li H, Hanakawa Y, et al. Genetic and functional characterization of human pemphigus vulgaris monoclonal autoantibodies isolated by phage display. *J Clin Invest* 2005;115:888–99.
- [15] Ishii K, Lin C, Siegel DL, Stanley JR. Isolation of pathogenic monoclonal anti-desmoglein 1 human antibodies by phage display of pemphigus foliaceus autoantibodies. *J Invest Dermatol* 2008;128:939–48.
- [16] Qian Y, Diaz LA, Ye J, Clarke SH. Dissecting the anti-desmoglein autoreactive B cell repertoire in pemphigus vulgaris patients. *J Immunol* 2007;178:5982–90.
- [17] Ishii K, Amagai M, Hall RP, Hashimoto T, Takayanagi A, Gamou S, et al. Characterization of autoantibodies in pemphigus using antigen-specific enzyme-linked immunosorbent assays with baculovirus-expressed recombinant desmogleins. *J Immunol* 1997;159:2010–7.
- [18] Driver DJ, McHeyzer-Williams LJ, Cool M, Stetson DB, McHeyzer-Williams MG. Development and maintenance of a B220- memory B cell compartment. *J Immunol* 2001;167:1393–405.
- [19] Tsunoda K, Ota T, Aoki M, Yamada T, Nagai T, Nakagawa T, et al. Induction of pemphigus phenotype by a mouse monoclonal antibody against the amino-terminal adhesive interface of desmoglein 3. *J Immunol* 2003;170:2170–8.
- [20] Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. Predominant autoantibody production by early human B cell precursors. *Science* 2003;301:1374–7.
- [21] Cook GP, Tomlinson IM. The human immunoglobulin VH repertoire. *Immunol Today* 1995;16:237–42.
- [22] Collins AM, Sewell WA, Edwards MR. Immunoglobulin gene rearrangement, repertoire diversity, and the allergic response. *Pharmacol Ther* 2003;100:157–70.

- [23] Kuwana M, Nomura S, Fujimura K, Nagasawa T, Muto Y, Kurata Y, et al. Effect of a single injection of humanized anti-CD154 monoclonal antibody on the platelet-specific autoimmune response in patients with immune thrombocytopenic purpura. *Blood* 2004;103:1229–36.
- [24] Shokrgozar MA, Sam MR, Amirkhani A, Shokri F. Frequency analysis of HBsAg-specific B lymphocytes in high-responder individuals to recombinant hepatitis B vaccine: comparison of LDA and ELISPOT assays. *Scand J Immunol* 2006;64:536–43.

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