T Lymphocytes from a Subset of Patients with Pemphigus Vulgaris Respond to Both Desmoglein-3 and Desmoglein-1

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Pemphigus vulgaris and pemphigus foliaceus are cutaneous autoimmune diseases characterized by intraepithelial blisters and autoantibodies to desmosomal glycoproteins. The antigens recognized by pemphigus vulgaris and pemphigus foliaceus autoantibodies are desmoglein-3 (Dsg3) and desmoglein-1 (Dsg1), respectively. Dsg3 and Dsg1 are members of the desmoglein subfamily of the cadherin supergene family of cell adhesion molecules. It has been well documented that a subset of pemphigus vulgaris sera have IgG reactivity to both Dsg1 and Dsg3, suggesting that Dsg1 may also participate in the autoimmune response of these patients. The cellular mechanisms of T cell autoimmunity in these patients, however, are completely unknown. In this study, we tested the proliferative responses of T lymphocytes from eight pemphigus vulgaris patients after incubation with Dsg3 and Dsg1 fusion proteins. The sera of four of these PV patients showed reactivity with both Dsg1 and Dsg3, whereas the remaining four reacted only with Dsg3. We found that T cells obtained from those patients that exhibited the combined Dsg1/Dsg3 autoantibody reactivity showed a proliferative response after exposure to either Dsg1 or Dsg3 fusion proteins. The cellular responses to both of these recombinant proteins were highly specific and restricted to the CD4-positive T cell population. T cells from pemphigus vulgaris patients with no anti-Dsg1 serum reactivity showed a proliferative response to Dsg3, but not to Dsg1. The Dsg1 fusion protein used in this study has minimal sequence homology with Dsg3. Thus, this study provides the first evidence that T cells from a subset of pemphigus vulgaris patients respond to both Dsg1 and Dsg3. *Key words: autoantibodies/autoimmunity/bullous diseases/desmosome. J Invest Dermatol 109:734–737, 1997*

Pemphigus vulgaris (PV) and pemphigus foliaceus (PF) are autoantibody-mediated cutaneous diseases characterized by intraepidermal blisters and pathogenic anti-desmosomal autoantibodies (Beutner and Jordon, 1964; Lever, 1965; Stanley *et al*, 1984). Based on clinical, histologic, and immunologic findings, PV and PF are distinct autoimmune diseases. In PV, the disease affects skin and mucosal epithelia and acantholysis is observed in the suprabasilar layer, whereas in PF, the disease is epidermal specific and cell–cell detachment occurs within the subcorneal layer (Civatte, 1943; Lever, 1965). PV and PF anti-epidermal autoantibodies were first shown to be pathogenic by passive transfer experiments using neonatal BALB/C mice (Anhalt *et al*, 1982; Roscoe *et al*, 1985; Futamura *et al*, 1989). These murine models reproduced the classic clinical and histologic features of these two human diseases. The predominant IgG isotype of the pathogenic anti-epidermal autoantibodies in both diseases is IgG4 (Jones *et al*, 1988; Rock *et al*, 1989; Allen *et al*, 1993; Bhol *et al*, 1994).

The autoantigens recognized by PV and PF autoantibodies have been identified as desmoglein-3 (Dsg3) (Stanley *et al*, 1984; Amagai *et al*, 1991) and desmoglein-1 (Dsg1) (Stanley *et al*, 1984; Wheeler *et al*, 1991), respectively. The 160-kDa Dsg1 and the 130-kDa Dsg3 are transmembrane desmosomal glycoproteins, and both are members of the desmoglein subfamily of the cadherin supergene family (Buxton and Magee, 1992). It has been reported recently that recombinant Dsg1 and Dsg3 generated in the baculovirus expression system are capable of adsorbing pathogenic autoantibodies from the sera of the respective patients (Amagai *et al*, 1994, 1996). These experiments provide strong evidence that autoantibodies against Dsg1 and Dsg3 participate in the immune injury of epidermis and mucosal tissues in patients with PF and PV, respectively.

In addition to anti-Dsg3 autoantibodies, ≈50% of PV sera recognize Dsg1 (Eyre and Stanley, 1988; Amagai *et al*, 1994; Emery *et al*, 1995). The significance of these anti-Dsg1 autoantibodies in the pathogenesis of PV remains unknown. Interestingly, certain patients appear to show a clinical, histologic, and immunologic transition from PV to PF (Iwatsuki *et al*, 1991; Kawana *et al*, 1994; Hashimoto *et al*, 1995). Sera from this particular subset of PV patients react with the extracellular domain of Dsg1 by immunoblotting, immunoprecipitation, and immunofluorescence (IF) techniques (Emery *et al*, 1995; Kowalczyk *et al*, 1995).

In this communication, we report that T cells from a subset of four PV patients whose sera immunoprecipitate Dsg1, proliferate in response to a segment of Dsg1 that shares minimal homology with the corresponding region of Dsg3. The proliferative response of T cells to Dsg1 is specific and reproducible. Our data therefore provide the first direct evidence that both Dsg1 and Dsg3 are involved in the autoimmune responses in a particular subset of PV patients that share in common anti-Dsg1 and anti-Dsg3 autoantibodies. Furthermore, we demonstrate that the Dsg1-specific T cells from this subset of PV patients exhibit the CD4, but not the CD8, cell marker. Future

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Abbreviations: BP, bullous pemphigoid; CP, cicatricial pemphigoid; Dsg1, desmoglein-1; Dsg3, desmoglein-3; FP-K, fusion protein K; GST, glutathione-S-transferase; PBMC, peripheral blood mononuclear cells; PF, pemphigus foliaceus; PV, pemphigus vulgaris.

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Table I. Dsg1 and Dsg3 can stimulate T cells from PV patients with a combined anti-Dsg1/Dsg3 autoantibody response

Patients	Disease duration	Immunoprecipitation[a]		T cell proliferation[b]					
		Dsg1	Dsg3	Dsg1 (FP-K)	Dsg3-A[c]	Dsg3-B[c]	Dsg3-C[c]	SΔ1	GST
EG	2 y	+	+	30.6	27.5	13.0	24.0	(-)	(-)
WAF	2 y 5 mo	+	+	12.0	85.0	45.6	28.0	(-)	(-)
NW	1 y 9 mo	(-)	+	(-)	17.0	6.0	6.3	(-)	(-)
LS	5 y 8 mo	+	+	12.0	10.0	8.0	9.0	(-)	(-)
UP	5 mo	(-)	+	(-)	13.0	16.0	10.0	(-)	(-)
EF	2 y 7 mo	(-)	+	(-)	5.0	4.0	3.0	(-)	(-)
WE	3 y 2 mo	+	+	10.0	4.0	(-)	7.0	(-)	(-)
ML	1 y 8 mo	(-)	+	(-)	6.0	5.0	3.0	(-)	(-)

[a]The presence of antibodies to Dsg1 and Dsg3 were detected by immunoprecipitation as described in *Materials and Methods*.
[b]T cell proliferation assays were applied to determine the responses of T lymphocytes to Dsg1, Dsg3, and SΔ1 fusion proteins. Data are presented in terms of the stimulation index. A stimulation index equal to or greater than three is considered a positive response. T cells that do not respond to stimulation are indicated as (-).
[c]Data were published previously (Lin *et al*, 1997b).

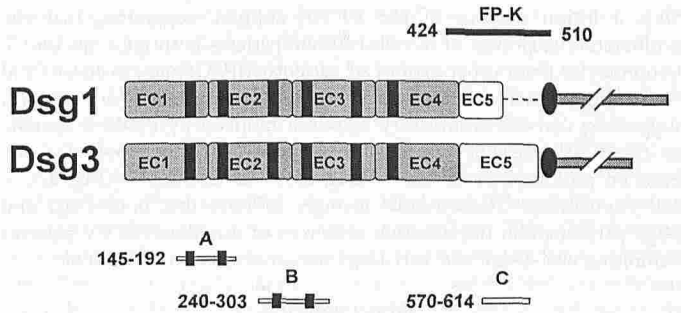

Figure 1. Dsg1 and Dsg3 fusion proteins. Both Dsg1 and Dsg3 contain five major cadherin-like domains on the extracellular portion. The *black strips* are the Ca^{2+} binding sites. The locations of Dsg1 and Dsg3 fusion proteins used in this study are shown as horizontal bars.

characterization of T cell responses in these patients will help to elucidate the mechanism(s) responsible for disease progression and may shed light on the transition of PV to PF.

MATERIALS AND METHODS

PV patients and controls Sera and peripheral blood mononuclear cells (PBMC) were obtained from eight well-characterized PV patients, who were followed at the Department of Dermatology Clinics of the Medical College of Wisconsin. The PV patients included in this study showed positive direct and indirect IF studies. Patients with other cutaneous autoimmune disease, such as bullous pemphigoid (BP) (n = 3), cicatricial pemphigoid (CP) (n = 2), and psoriasis (n = 4), were included along with normal volunteers (n = 8) as controls.

Preparation of Dsg3 and Dsg1 fusion proteins To evaluate the response of T cells of PV patients to Dsg1, we generated a bacterial fusion protein (designated FP-K; **Fig 1**) containing a portion of the E5 region of Dsg1, which shows minimal sequence homology with Dsg3 (see *Results*). Regions E1–E4 of Dsg1, which exhibit 55.9% sequence identity with Dsg3 (Amagai *et al*, 1991), were avoided to minimize potential cross-reactivity problems. To generate fusion protein K (FP-K), the Dsg1 cDNA segment encoding amino acids 424–510 was polymerase chain reaction amplified and subcloned into the bacterial expression vector pGEX2T (Pharmacia, Piscataway, NJ) as previously described (Liu *et al*, 1995). The resulting fusion protein consisted of glutathione S-transferase (GST) fused to the Dsg1 peptide made up of the following sequence: RTCTGTININIQSFGNDDRTNTEPNTKITTNTGRQESTSSTNYDTST-TSTDSSQVYSSEPGNGAKDLLSDNVHFGPAGIGLLIMGFL. Also used in this study were GST-Dsg3 fusion proteins A, B, and C containing Dsg3 amino acids 145–192, 240–303, and 570–614, respectively. The Dsg3 fusion proteins were also generated using the pGEX expression system and were described in a recent publication (Lin *et al*, 1997b; **Fig 1**). The recombinant Dsg1 and Dsg3 proteins were expressed in *Escherichia coli* strain DH5α and purified by glutathione-agarose affinity chromatography (Liu *et al*, 1992). The purified fusion proteins were dialyzed against phosphate-buffered saline, concentrated by ultrafiltration, and filter-sterilized. The protein concentration was determined by Bradford protein assays (Bio-Rad, Hercules, CA).

Immunoprecipitation of Dsg1 and Dsg3 The presence of anti-Dsg3 and anti-Dsg1 autoantibodies in sera from PV patients was detected by immunoprecipitation as described (Labib *et al*, 1990). The ectodomain of Dsg3 used in this experiment was prepared using a baculovirus expression system (Amagai *et al*, 1994), whereas Dsg1 immunoreactive tryptic fragments were obtained from bovine snout epidermis following procedures described by our laboratory (Olague-Alcala *et al*, 1994). Both Dsg3 and the tryptic fragments of Dsg1 were labeled with ^{125}I following the chloramine T method (Calvanico *et al*, 1991; Olague-Alcala and Diaz, 1993).

Purification of peripheral blood mononuclear cells and isolation of CD^{4+} and CD^{8+} T cells PBMC were isolated by Ficoll-Hypaque (Pharmacia) density gradient separation (Vila *et al*, 1995). T cells were then purified by E-rosetting using 2-amino-ethylisothiouronium bromide (Sigma, St. Louis, MO)-treated sheep red blood cells (Colorado Serum, Denver, CO) (Indiverti *et al*, 1980). CD^{4+} or CD^{8+} T lymphocytes were further purified from the total T cell population by negative selection using a magnetic cell sorter (Miltenyi Biotec, Auburn, CA) combined with anti-CD8 (Biosource, Camarillo, CA) or anti-CD4 antibodies (Biosource) (Miltenyi *et al*, 1989). The purity of the CD^{4+} and CD^{8+} cell isolates was greater than 98% as determined by fluorescence-activated cell sorter analysis (not shown). The purified T cells were washed with medium three times, and resuspended in RPMI1640 medium supplemented with 10% human AB serum (NABI, Miami, FL) for the T cell proliferation assays.

T cell proliferation assays T cell responses to Dsg1 and Dsg3 fusion proteins were determined by proliferation assays as described (Lin *et al*, 1997b). Briefly, T cells at a density of 10^5 per ml were cultured with 10^5 per ml of irradiated autologous PBMC as antigen-presenting cells in wells of 96-well U-bottom plates for 7 d. Phytohemagglutinin (Sigma) at 0.25 μg per ml or IL-2 at 10 U per ml served as positive controls in all T cell proliferation experiments. The GST-BP180 fusion protein SΔ1 (Giudice *et al*, 1993, 1994) was also included in this study. Cells in individual wells were pulsed with 1 μCi of [^{3}H]thymidine (ICN) during the last 18 h of incubation and then harvested using an automated cell harvester (Inotech Biosystems, Lansing, MI). The proliferation of T cells was determined by measuring the [^{3}H]thymidine uptake on a β-counter (Wallac, Gaithersburg, MD). Data were presented as average cpm ± SD or stimulation index (cpm of cells treated with fusion proteins ÷ cpm of cells treated with GST at the same concentration). A stimulation index equal to or greater than three was considered a positive response.

RESULTS

Sera from a subset of PV patients immunoprecipitate Dsg3 and Dsg1 The sera from the eight PV patients included in this study were analyzed by immunoprecipitation for reactivity with Dsg1 and Dsg3, and the results are presented in **Table I**. For use in this analysis, a recombinant protein encompassing the entire extracellular domain of Dsg3 was produced in the baculovirus system. This 66-kDa protein was immunoprecipitated by all eight PV sera, but not by any of the control sera. A radio-iodinated epidermal preparation containing a 45-kDa tryptic fragment of bovine Dsg1 was also used to test the PV and control sera. This 45-kDa Dsg1 peptide, which has previously been shown to be recognized by all PF sera and a subset of PV sera (Olague-Alcala *et al*, 1994), was immunoprecipitated by four of the eight PV

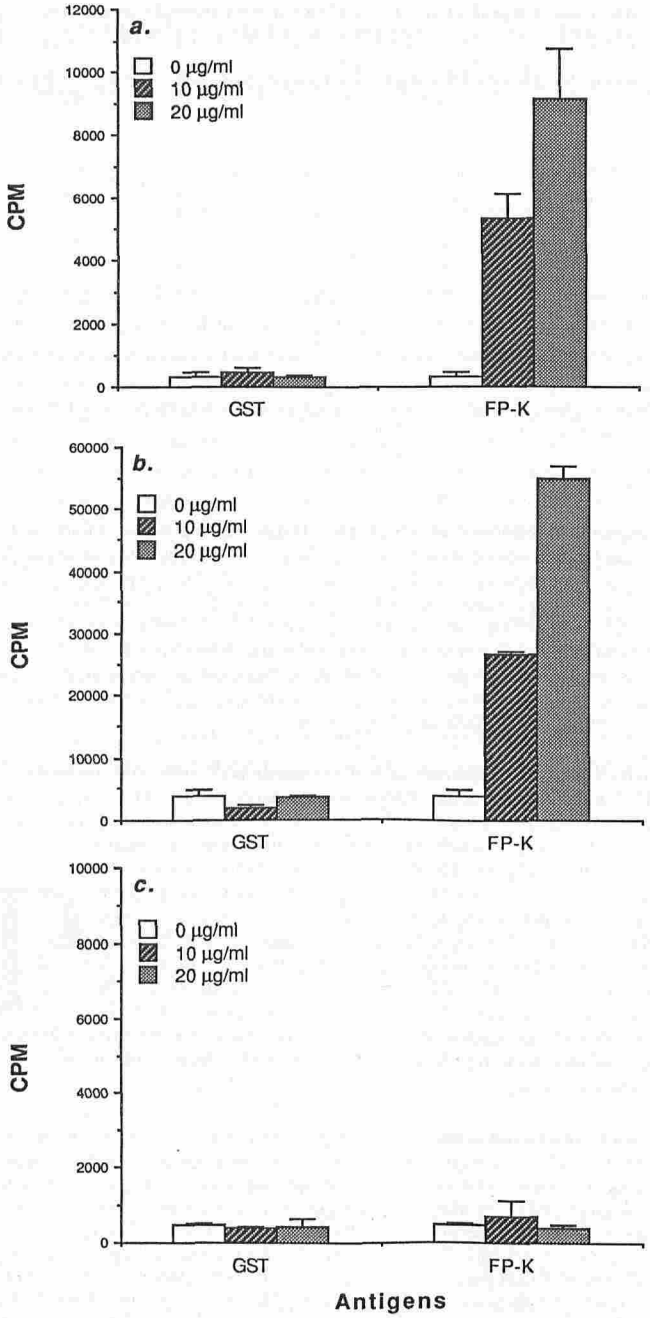

Figure 2. T lymphocytes from a subset of PV patients proliferate in response to Dsg1 FP-K. The proliferative responses of T cells from a representative PV patient to the FP-K were examined as described in *Materials and Methods*. The responses of the total T cell population (*a*), CD^{4+} T cells (*b*), and CD^{8+} T cells (*c*) to this Dsg1 peptide were determined by [^{3}H]thymidine incorporation. Data were expressed as average cpm + SD.

sera in this study. None of the control sera reacted with this Dsg1 peptide. Thus, these results demonstrate unequivocally that sera from these four PV patients react with both Dsg1 and Dsg3.

T lymphocytes from a subset of PV patients respond to a Dsg1 fusion protein Previously, we have identified three peptide segments on the ectodomain of Dsg3 that are recognized by T cells from PV patients (Lin *et al*, 1997b). These Dsg3 peptides induce proliferation of T cells from PV patients in a concentration-dependent manner. To further examine whether T lymphocytes from these PV patients respond to Dsg1, proliferation assays were performed using FP-K that encompasses amino acids 424–510 of Dsg1. This Dsg1 peptide was chosen because its amino acid sequence was highly divergent from that of the corresponding region of Dsg3. Based on sequence comparisons between the FP-K insert and the entire human Dsg3 ectodomain using FASTA and BESTFIT [Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI], the longest stretch of identical residues was three. After allowing for conservative substitutions, the longest contiguous stretch of similarity between these two protein sequences was seven. The overall similarity and identity values for these two sequences, allowing for the introduction of gaps, were 41% and 19.5%, respectively.

T cells from a subset of PV patients showed a proliferative response to the FP-K fusion protein, and this response was shown to be concentration dependent. The results from a representative patient are shown in **Fig 2*a***. Of the eight PV patients tested, T cell proliferation to FP-K was observed in samples from four, all of whom also had circulating anti-Dsg1 autoantibodies, as determined by immunoprecipitation. The proliferative response of PV T cells to FP-K is presented in terms of the stimulation index and is shown in **Table I**. This Dsg1 reactivity was not seen in individuals whose sera only recognized Dsg3. T cells from this subset of PV patients did not respond to GST or to SΔ1, a fusion protein of the BP180 antigen, suggesting that the proliferative responses of T cells of these patients is antigen specific. T lymphocytes from other groups of patients (BP, CP, and psoriasis) and control individuals did not respond to this Dsg1 peptide (not shown), suggesting that the stimulatory response induced by FP-K is specific to these PV patients. Moreover, we found that this peptide only induced proliferation of CD^{4+} (**Fig 2*b***), and not CD^{8+} (**Fig 2*c***), T cell populations. These results strongly indicate that both Dsg1 and Dsg3 participate in the immune responses of this subset of PV patients exhibiting anti-Dsg1 and anti-Dsg3 autoantibodies in their sera.

DISCUSSION

It has been well documented that ≈50% of PV sera possess, in addition to anti-Dsg3 autoantibodies, anti-Dsg1 autoantibodies. The vast majority of these patients show typical clinical and histologic features of PV, although on rare occasions patients may undergo a shift from PV to PF during the course of their disease (Koulu and Stanley, 1988; Iwatsuki *et al*, 1991). Based on the high sequence homology (55.9% identity over most of the ectodomain) exhibited by Dsg1 and Dsg3, it has been hypothesized that autoantibodies produced by this subset of PV patients react with a set of epitopes shared by Dsg1 and Dsg3; however, it was not previously known whether T cells from these PV patients specifically respond to Dsg1.

The data presented here clearly demonstrate that T cells from a subset of PV patients, showing both anti-Dsg1 and anti-Dsg3 autoantibodies in their sera, proliferate when incubated with a Dsg1 fusion protein that encompasses amino acids 424–510. Based on the very low sequence similarity found between the FP-K insert and Dsg3, as described in the *Results*, it is unlikely that T cells that respond to this 87 amino acid stretch of Dsg1 are also reactive with an epitope on Dsg3. Furthermore, this Dsg1 segment has been shown to specifically activate T cells from patients with PF (n = 3) or fogo selvagem (n = 8) (Lin *et al*, 1997a), supporting the notion that this protein may possess the necessary epitopes to stimulate PF T cells.

The expression of certain HLA-DR alleles by these patients as well as the clinical presentation and the reactivity of T cells and autoantibodies with Dsg1 showed no correlation; however, this might, in part, be due to the small number of patients that were investigated. It is also unclear at this time whether the development of immune responses to Dsg1 in these PV patients is related to the duration and/or severity of disease. There is, however, a general trend that patients with longer duration of PV in this study (over 2–3 y) are more likely to exhibit a T cell response against Dsg1 compared with patients who had the disease for a shorter period (under 2 y; **Table I**).

The specificity of the T cell response to the FP-K Dsg1 peptide was substantiated by showing that T cells from all eight PV patients were unresponsive to recombinant GST, the carrier protein for the Dsg1 fusion protein, or SΔ1, a GST fusion protein containing a 42 amino acid segment of the bullous pemphigoid antigen BP180. In addition, T cells from other control groups, such as healthy individuals

and BP, CP, and psoriasis patients, did not respond when incubated with Dsg1 or control antigens, further documenting the specificity of the Dsg1-induced T cell response in these PV patients. Although the Dsg1 responsive T cells from these patients were shown to be CD^{4+} positive, the nature of the cytokine profile that they secrete remains under investigation. It is also unknown whether the Dsg1-responsive T cells in this subset of PV patients are pathogenically relevant.

The epitopes of Dsg1 and Dsg3 antigens recognized by PV sera are known to be localized in the desmosomal core (Karpati *et al*, 1993; Shimizu *et al*, 1995). Moreover, it has been reported that sera from certain PV patients also possess reactivity to desmocollins, another group of desmosomal core glycoproteins (Hashimoto *et al*, 1995). These findings suggest that these desmosomal antigens are exposed to the immune system in PV patients once the acantholytic process splits these organelles. Thus, the T and B cellular and humoral response to Dsg3 and Dsg1, in the particular subset of PV patients reported in this paper, may represent concurrent autoimmune reactions to desmosomal core antigens that are uncovered during the course of the epidermal disease. The initial target epitope that triggers the autoimmune response, however, remains unknown. It is expected that characterization of this initial epitope would be a difficult task due to the diversity of the T and B cell responses to Dsg3 and Dsg1 epitopes and other desmosomal antigens. Because the sera of all PV patients recognize Dsg3 by immunoprecipitation, it may be assumed that the initial T cell immune response is directed to an epitope on Dsg3. In the course of the disease, this autoimmune response may spread to involve other Dsg3 epitopes and perhaps other antigen(s) such as Dsg1 and desmocollins that are located within the same cellular organelles. This "determinant spreading" phenomenon of immune responses has been observed in other autoimmune diseases such as experimental autoimmune encephalomyelitis and spontaneous insulin-dependent diabetes in the nonobese diabetic mouse (Lehmann *et al*, 1992; Lehmann *et al*, 1993; Kaufman *et al*, 1993). Because both Dsg1 and Dsg3 co-localize to the epidermal desmosomal core, it raises the possibility that T cells from PV patients may recognize Dsg3 and Dsg1 in a stepwise manner as stated by the determinant spreading theory.

This study demonstrates the complexity of autoimmune responses in a particular subset of PV patients and will allow us to further characterize the T cell responses in these patients.

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