Desmocollins I and II Are Recognized by Certain Sera from Patients with Various Types of Pemphigus, Particularly Brazilian Pemphigus Foliaceus

Marian Dmochowski, Takashi Hashimoto, David R. Garrod, and Takeji Nishikawa

Department of Dermatology (MD,TH,TN), Keio University School of Medicine, Tokyo, Japan; and Department of Cell and Structural Biology (DRG), School of Biological Sciences, University of Manchester, Manchester, U.K.

Recently, it has been shown that desmoglein, pemphigus foliaceus target antigen, and a 130-kD pemphigus vulgaris antigen belong to the cadherin family of cell adhesion molecules. We tried to determine whether desmocollins I/II, other cadherin-like transmembranous glycoproteins present in desmosomes, are also recognized by pemphigus autoantibodies of the IgG class. We examined 16 pemphigus vulgaris sera, 15 pemphigus foliaceus sera, 15 Brazilian pemphigus foliaceus sera, five bullous pemphigoid sera, and 65 normal sera. Four (25%) pemphigus vulgaris sera, one (7%) pemphigus foliaceus serum, eight (53%) Brazilian pemphigus foliaceus sera, and three (5%) normal sera reacted with desmocollins I/II on immunoblots of bovine desmosome preparation. The affinity-purified desmocollins I/II pemphigus autoantibodies were shown to bind the epidermal cell surface by indirect immunofluorescence. Immunoblot analysis revealed one pemphigus vulgaris serum, one Brazilian pemphigus foliaceus serum, and one normal serum recognizing a recombinant protein produced by a desmocollin cDNA clone. Moreover, immunoblot analysis of reactivity of a Brazilian pemphigus foliaceus serum with recombinant proteins produced by deletion mutants of the desmocollin cDNA clone showed that the extracellular portion of desmocollin is immunogenic in this pemphigus patient.

We conclude that desmocollins I/II are recognized by certain sera from patients with various types of pemphigus, particularly Brazilian pemphigus foliaceus. However, the significance of this reactivity remains to be defined. *J Invest Dermatol 100:380–384, 1993*

Pemphigus is an autoimmune blistering disease that can be subdivided into two major subtypes: pemphigus vulgaris (PV) and pemphigus foliaceus (PF). Brazilian (endemic) pemphigus foliaceus (BPF) (fogo selvagem) is considered to be a variant of pemphigus foliaceus [1]. PV antigen, which is defined by autoantibodies from PV patients, has been characterized by immunoprecipitation and immunoblotting as a 130-kD glycoprotein, whereas PF antigen, detected by the same techniques, is a 160-kD or 150-kD desmosomal glycoprotein (desmoglein) [2,3]. Recently, it has been established that these proteins belong to the cadherin family of calcium-dependent cell adhesion molecules [4–8]. Desmocollins (DC) I/II, a pair of 115-kD and 107-kD proteins [9], have also been shown to be cadherin-like glycoproteins present in desmosomes [10–14]. In our previous immunoblot study of BPF, we have noticed that a considerable number of BPF sera reacted with a pair of 115-kD and 105-kD proteins [15], which we later supposed were DC I/II. Additionally, we have recently shown that IgA anti-cell surface autoantibodies found in certain atypical cases with vesiculopustular lesions seemed to react with DC I/II [16].

Therefore, in this introductory study, we wanted to determine if DC I/II are recognized by pemphigus antibodies of the IgG class and which portion of these molecules is immunogenic in pemphigus patients.

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Reprint requests to: Dr. T. Hashimoto, Department of Dermatology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku, Tokyo 160, Japan.

Abbreviations:
BP: bullous pemphigoid
BPF: Brazilian pemphigus foliaceus
DC: desmocollin(s)
IF: immunofluorescence
PF: pemphigus foliaceus
PV: pemphigus vulgaris
SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis

MATERIALS AND METHODS

Sera We examined 16 PV, 15 PF, and 15 BPF sera. Five bullous pemphigoid (BP) sera containing anti–basement membrane zone antibodies at a titer of greater than 1:40 as determined by indirect immunofluorescence (IF), and 65 normal human sera were used as controls. All pemphigus sera contained anti-cell surface antibodies at a titer of greater than 1:40 as determined by indirect IF on normal human epidermis. None of control sera contained anti-cell surface antibodies. All sera were stored at −80°C and briefly at 4°C in the presence of 0.1% NaN_3 during the experiments.

Monoclonal Antibodies We used anti–DC I/II monoclonal antibody (52-3D) [10] recognizing their cytoplasmic regions; anti-desmoglein monoclonal antibody (32-2B), which recognizes its cytoplasmic region [17]; and anti-desmoplakins I/II monoclonal antibody (11-5F) [18].

Construction and Isolation of an Expression Clone of DC II cDNA, and Preparation of its Deletion Mutants All reagents, except for those used in deletion experiment, were purchased from Toyobo Co., Osaka, Japan. DC II cDNA clone (CN35) was obtained from a bovine nasal epidermal λgt11 cDNA library [14]. To

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produce DC II recombinant protein efficiently, 2.77-kb cDNA fragment (designated CN35-XbaI) was obtained by digestion of CN35 at XbaI restriction sites in the precursor sequence and a little downstream to the stop codon, and subcloned in-frame into pUC18. Therefore, CN35-XbaI produced recombinant protein containing entire mature DC II. To produce unidirectional (from 5′ end encoding extracellular amino-terminus of DC II) deletions (Deletion Kit for Kilosequence; Takara Biomedicals, Kyoto, Japan), the pUC18-CN35-XbaI was first double digested with KpnI (unique 3′ overhang restriction site) and BamHI (unique 5′ overhang restriction site lying between the 5′ end of CN35-XbaI insert and KpnI restriction site). Then, double-digested DNA was treated with exonuclease III for various time intervals, followed by treatment with Mung Bean nuclease, and finally ligated with T4 DNA ligase to recircularize. Deletion mutants of CN35-XbaI were transfected into *Escherichia coli* strain XL1-Blue. The size of inserts of deletion mutants of CN35-XbaI clone was determined on 0.7% agarose gel electrophoresis after inserts were cut out by double digestion with EcoRI and SalI.

Production of DC II Recombinant Proteins The XL1-Blue cells transformed with CN35-XbaI or its deletion mutants were cultured at 37°C for 3.5 h in LB medium and induced by an addition of 1.5 mM isopropyl β-D-thiogalactopyranoside. After 3.5 h further culture the cells were harvested, resuspended in 50 mM Tris-HCl buffer (pH 8.0) with 1 mM ethylenediaminetetraacetic acid, and treated with lysozyme. After the extraction with deoxycholic acid and treatment with DNase I, the lysates were centrifuged at 13,000 × *g* and the pellets boiled in Laemmli's sample buffer with 5% β-mercaptoethanol before being subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Immunoblot Analysis Dermoepidermal separation with dispase, extraction of normal human epidermis, and preparation of bovine desmosome sample were performed according to the procedures described previously [3,15,19]. Dispase-treated normal human epidermal extract (80 μg protein/lane), bovine desmosome preparation (6 μg protein/lane), recombinant protein of CN35-XbaI (RP1), and recombinant proteins of CN35-XbaI deletion mutants (RP2-5) were run on SDS-PAGE according to Laemmli's method [20] using 7% separating gel (for human epidermal extract, bovine desmosome preparation, and RP1 analysis with sera or monoclonal antibodies) or 10% separating gel (for RP1-5 analysis with BPF2 serum), and electrophoretically transferred to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) [21]. For immunostaining, blots were first blocked with 3% skim milk in Tris-HCl–buffered saline (pH 8.0) and then reacted with sera diluted 1:40 (in experiments with human epidermal extract and bovine desmosome preparation), with sera diluted 1:20 and preabsorbed with equal volume of lysate of plasmid-free XL1-Blue for 1 h at room temperature to reduce background staining (in experiments with RP1-5), or with monoclonal antibodies diluted 1:200. Peroxidase-conjugated anti-human IgG rabbit antiserum (DAKO) diluted 1:100 or peroxidase-conjugated rabbit anti-mouse immunoglobulins antiserum (DAKO) diluted 1:100 were used as a secondary antibody. Color was developed by 4-chloro-1-naphthol in the presence of hydrogen peroxide.

Affinity Purification of DC I/II IgG Affinity purification of DC I/II IgG was performed mainly according to the previously described methods [4,22]. Briefly, horizontal strips of nitrocellulose containing the bovine DC I/II and control strips of equal width below the area where the DC I/II were located were cut out, incubated with a BPF serum or a PV serum, and washed. Bound antibodies were eluted with sodium citrate buffer (pH 3.2), immediately neutralized, dialyzed against phosphate-buffered saline, and concentrated by ultrafiltration (Centriprep 10, Amicon, Danvers, MA).

Indirect IF The concentrated eluates were used to determine whether the affinity-purified DC I/II IgG bind the epidermal cell surface. Indirect IF using either normal human skin or bovine muzzle epidermis as a substrate and fluorescein isothiocyanate–conjugated rabbit immunoglobulins to human IgG (DAKO, Copenhagen, Denmark) as a secondary antibody was performed according to the standard method.

RESULTS

Immunoblot Analysis of Human Epidermal Extract All PV sera recognized the 130-kD human PV antigen. Of 15 PF sera, seven labeled the 150-kD antigen (desmoglein) and one labeled the 130-kD PV antigen. Of 15 BPF sera, seven recognized desmoglein and three labeled the 130-kD PV antigen. All BP sera reacted weakly with the 230-kD BP antigen, but failed to recognize the 180-kD BP antigen, desmoglein, the 130-kD PV antigen, and DC I/II. None of 65 control normal sera recognized any specific epidermal proteins (Table I). Although anti–DC I/II monoclonal antibody labeled weakly a pair of 110-kD and 100-kD proteins (presumably human DC I/II), this pair was not labeled by any sera, including those reacting strongly with bovine DC I/II in desmosome preparation (see below).

Immunoblot Analysis of Bovine Desmosome Preparation As shown in Table I and Fig 1, of 16 PV sera 12 labeled the 135-kD bovine PV antigen, eight recognized desmoglein, and four (25%) detected a pair of 115-kD and 105-kD proteins. These proteins were considered to be DC I/II, because anti–DC I/II monoclonal antibody and 115-kD/105-kD protein–positive sera identified co-migrating bands. All PF sera recognized desmoglein and one (7%) PF serum detected DC I/II. Of 15 BPF sera, 13 labeled desmoglein and eight (53%) detected DC I/II. Of 65 control normal sera, seven recognized desmoglein and three (5%) DC I/II. Virtually all desmoglein- and/or DC I/II–positive normal sera reacted with these proteins much more weakly than pemphigus sera did. None of five BP sera recognized any specific bands, including the 230-kD and 180-kD BP antigens, desmoglein, the 135-kD bovine PV antigen, and DC I/II.

The Analysis of the Affinity-Purified DC I/II IgG We obtained the affinity-purified DC I/II IgG from either the BPF serum labeling desmoglein and DC I/II on immunoblots of bovine desmosome sample or the PV serum reacting with DC I/II on immunoblots of bovine desmosome preparation and the 130-kD human PV antigen. The affinity-purified DC I/II IgG from both cases bound DC I/II on immunoblots of bovine desmosome sample (Fig 2*A*). Furthermore, the affinity-purified DC I/II IgG from the BPF serum did not show the reactivity with desmoglein, indicating that DC I/II and desmoglein do not share common epitopes. Control eluates did not bind any proteins on immunoblots (Fig 2*A*). Indirect IF on either normal human skin or bovine muzzle epidermis demonstrated that the affinity-purified DC I/II IgG prepared from either the BPF serum or the PV serum bound the epidermal cell surface (Fig 2*B*). Control eluates prepared from both sera did not show the epidermal cell surface staining (Fig 2*C*).

Immunoblot Analysis of RP1 (DC II Recombinant Protein Produced by Undeleted 2.77-kb CN35-XbaI Clone) Only anti–DC I/II monoclonal antibody reacted with RP1, whereas culture supernatant of parental cells for hybridoma, anti-desmoglein monoclonal antibody, and anti-desmoplakins I/II monoclonal antibody did not show any reactivity, confirming the specificity of RP1 (data not shown). The results of RP1 analysis with sera are shown in Table I and Fig 3. RP1 was recognized by one (6%) of 16 PV sera, one (7%) of 15 BPF sera, and one (1%) of 65 control sera. None of the PF sera recognized RP1. All sera that reacted with RP1 also recognized bovine DC I/II. None of BP sera labeled RP1. None of the control normal sera that recognized DC I/II in desmosome preparation and RP1 showed epidermal cell surface staining with indirect IF.

Table I. Reactivities of Sera in Immunoblotting of Human Epidermal Extract, Bovine Desmosome preparation, and DC II Recombinant Protein Produced by CN35-XbaI Clone (RP1)

Sera	Epidermal Extract			Desmosome Preparation			
	150 kD	130 kD	hDC[a]	150 kD	135 kD	bDC[b]	RP1
PV (n = 16)	0	16	0	8	12	4 (25%)	1 (6%)
PF (n = 15)	7	1	0	15	0	1 (7%)	0
BPF (n = 15)	7	3	0	13	0	8 (53%)	1 (7%)
BP (n = 5)	0	0	0	0	0	0	0
Control (n = 65)	0	0	0	7	0	3 (5%)	1 (1%)

[a] A pair of 110-kD and 100-kD proteins (human DC I/II).
[b] A pair of 115-kD and 105-kD proteins (bovine DC I/II).

Immunoblot Analysis of RP2-5 (DC II Recombinant Proteins Produced by Deletion Mutants of CN35-XbaI Clone) Four recombinant proteins produced by deletion mutants of CN35-XbaI clone having inserts of 1.95 kb (RP2), 1.50 kb (RP3), 1.35 kb (RP4), and 0.85 kb (RP5) were selected for analysis with BPF2 serum, which showed the strongest reactivity with DC I/II in bovine desmosome sample and with RP1. Both anti-DC I/II monoclonal antibody and BPF2 serum labeled RP1-4. Only RP5 (considered to correspond to the entire cytoplasmic region of DC II by the determination of the size of insert cDNA), although recognized by anti-DC I/II monoclonal antibody, was not labeled by BPF2 serum (Fig 4). These findings suggest that epitope(s) recognized at least by this particular serum is (are) localized on the extracellular region of DC I/II.

DISCUSSION

Recently, the heterogeneity of the antigen/antibody systems involved in the pathogenesis of pemphigus has been suggested on the basis of the production of IgM class anti-185-kD protein monoclonal antibody derived from a PV patient [23]. Moreover, it has been found that four polypeptides with molecular weights of 250 kD (desmoplakin I), 230 kD ("major" BP antigen), 210 kD (desmoplakin II), and 190 kD are immunoprecipitated by sera from patients with paraneoplastic pemphigus [24,25[, a mucocutaneous disease with some clinical features reminiscent of pemphigus. Such studies indicate that the further search for target antigens for the anti-epidermal cell surface autoantibodies is required.

In the present study, immunoblotting of bovine desmosome preparation shows that certain pemphigus sera clearly react with a pair of 115-kD and 105-kD proteins. Comparison with the reactivity of anti-DC I/II monoclonal antibody indicates that these proteins correspond to bovine DC I/II. Furthermore, the results of our affinity-purification studies indicate that DC I/II are indeed pemphigus antigens because the affinity-purified DC I/II IgG stained the epidermal cell surface. However, no sera examined by us showed any apparent reactivity with human DC I/II on immunoblots of the human epidermal extract. This discrepancy may be caused by the existence of quantitative as well as qualitative differences in the expression of epidermal proteins, including DC I/II, between epithelia from various species. It should be noted here that the usefulness of non-human epithelia for the detection of human pemphigus antibodies is well-established, e.g., it has been shown that the most sensitive substrates for the detection of PV and PF antibodies by indirect IF are monkey esophagus and guinea pig esophagus, respectively [26].

To confirm that some pemphigus sera react with DC I/II, we performed immunoblot analysis of recombinant protein produced

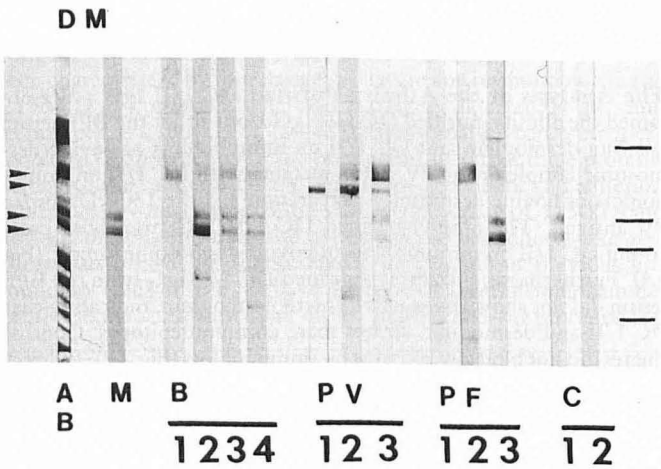

Figure 1. Immunoblot analysis of representative sera with the bovine desmosome preparation (DM). AB, amido black-stained lane; M, anti-DC I/II monoclonal antibody; B, BPF serum; PV, PV serum; PF, PF serum; C, control normal serum. *Arrowheads*, from top to bottom, the positions of the 150-kD protein (desmoglein), the 135-kD bovine PV antigen, and a pair of 115-kD and 105-kD proteins (this pair was regarded to be DC I/II because anti-DC I/II monoclonal antibody and 115-kD/105-kD protein-positive sera identified co-migrating bands). *Bars*, from top to bottom, the positions of molecular weight markers: 200 kD, 116 kD, 97 kD, and 66 kD. B1, B2, B3, and B4 sera recognized the 150-kD protein, whereas B2, B3, and B4 sera labeled additionally a pair of 115-kD and 105-kD proteins. PV1, PV2, and PV3 sera recognized the 135-kD PV antigen. PV2 and PV3 sera reacted also with the 150-kD protein, whereas PV3 serum reacted additionally with a pair of 115-kD and 105-kD proteins. PF1, PF2, and PF3 sera recognized the 150-kD protein. PF3 serum reacted also with a pair of 115-kD and 105-kD proteins. C1 serum labeled a pair of 115-kD and 105-kD proteins, whereas C2 serum did not show any reactivity.

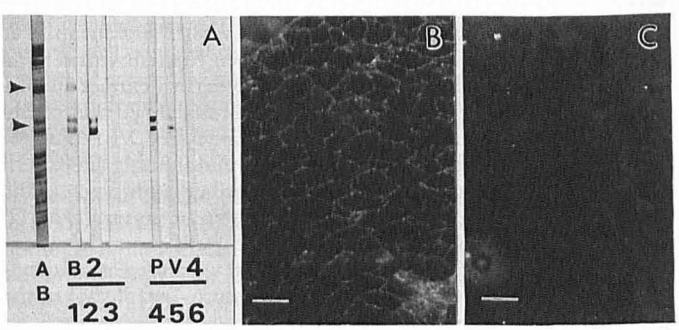

Figure 2. The analysis of the affinity-purified DC I/II IgG. Immunoblot analysis using bovine desmosome sample as an antigen source *(A)*. All abbreviations are the same as those in Fig 1. *Arrowheads*, from top to bottom, the positions of desmoglein and DC I/II. The B2 serum reacted with desmoglein and DC I/II *(lane 1)*, the PV4 serum reacted with DC I/II *(lane 4)*. This PV serum also recognized the 130-kD PV antigen with immunoblotting of the human epidermal extract. The affinity-purified DC I/II IgG prepared from B2 serum *(lane 2)* and PV4 serum *(lane 5)* reacted only with DC I/II. Control eluates prepared from B2 serum *(lane 3)* and PV4 serum *(lane 6)* did not show any reactivity. Indirect IF on bovine muzzle epidermis demonstrated that affinity-purified DC I/II IgG prepared from B2 serum bound the epidermal cell surface *(B)*, whereas control eluate prepared from the same serum did not bind the epidermis *(C)*. *Bars*, 10 μm.

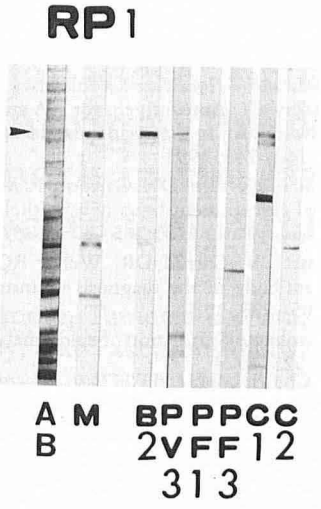

Figure 3. Immunoblot analysis of representative sera with the RP1. All abbreviations are the same as those in Fig 1. *Arrowhead,* the position of RP1. B2, PV3 and C1 sera recognized RP1, whereas PF1, PF3, and C2 sera did not. Lower bands seen in *lane M* were considered to be the degradation products of RP1.

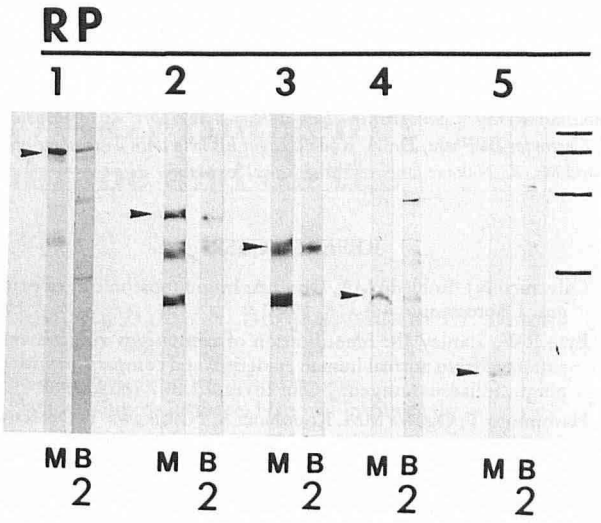

Figure 4. Immunoblot analysis of the BPF serum (B2) with a set of DC II recombinant proteins. RP1 indicates DC II recombinant protein produced by undeleted 2.77-kb CN35-XbaI clone. RP2-5 were produced by four deletion mutants of CN35-XbaI clone. The longest 1.95-kb clone encoded RP2, 1.50-kb clone encoded RP3, 1.35-kb clone encoded RP4, and the shortest 0.85-kb clone encoded RP5 (entire cytoplasmic region of RP1). *Lanes M,* anti-DC I/II monoclonal antibody. *Lanes B2,* BPF2 serum. *Arrowheads,* positions of DC II recombinant proteins. Lower bands seen in *lanes M* were considered to be the degradation products of recombinant proteins. *Bars,* from top to bottom, the positions of molecular weight markers: 116 kD, 97 kD, 66 kD, and 43 kD. Both anti-DC I/II monoclonal antibody and B2 serum recognized RP1-4. RP5 was labeled by anti-DC I/II monoclonal antibody but not by B2 serum.

by cDNA clone encoding entire mature DC II. This study indicates that certain pemphigus sera recognize DC II recombinant protein, although the frequency of this reactivity is very low. Thus, we hypothesize that the majority of DC I/II-positive sera react with conformation-dependent epitopes and/or sugar side chains on DC I/II. On the other hand, it has been suggested that the type of desmosomal cadherin expressed may vary as keratinization of skin takes place [27]. A DC-like 46/48-kD glycoprotein from human skin has been detected that, although it has homology with human DC, in fact has more homology with bovine DC [28]. Thus, there may be a set of DC-like molecules, and the antigen for the majority of DC I/II-positive sera may not be encoded by CN35 clone.

The reactivity of some normal control sera with DC I/II is puzzling. We hypothesize that certain normal human sera recognize DC-related molecules having common epitopes with typical DC. These epitopes may not be expressed in intact epidermis, which may be responsible for the lack of epidermal cell surface staining by the DC-positive normal human sera. Alternatively, such sera may contain non-specific antibodies to carbohydrate moiety of various glycoproteins, including desmoglein and DC I/II. These carbohydrate moiety-recognizing antibodies may bind to the desmosomal glycoproteins too weakly to be detected by indirect IF.

The low frequency of reactivity of pemphigus sera with the DC II recombinant protein, as well as the existence of a few DC I/II-positive normal sera, make the significance of anti-DC I/II antibodies in pemphigus uncertain at present. However, more than 50% of BPF sera examined in this study labeled DC I/II. It is interesting that BPF sera reacted with DC I/II much more frequently than sporadic PF sera despite the fact that both BPF and sporadic PF sera examined in this study contained roughly similar high titers of pemphigus antibodies. This finding suggests that the reactivity of some BPF sera with DC is not a function of antibody titer. Thus, we suggest that the possible role of anti-DC I/II autoantibodies in the pathogenesis of at least BPF should be defined.

In this study we also tried to determine which portion of DC I/II is immunogenic in pemphigus patients. Our results suggest that the extracellular region of these molecules contains epitope(s) for certain pemphigus antibodies. The question immediately arises regarding the exact nature of these epitopes. It has been found [29] that the epitopes for antibodies capable of blocking cadherin action are located in their amino-terminal 113 amino acid region. Recently, Amagai *et al* [30] have demonstrated that autoantibodies against the amino-terminal region of the PV antigen are pathogenic. Moreover, it has been shown [31] that the epitope for monoclonal antibody DECMA-1, capable of blocking E-cadherin function, is located close to the membrane-proximal part of its extracellular domain. The significant homology of DC I/II with typical cadherins [11-14,32] suggests that the amino-terminus of DC I/II and their membrane-proximal part of the extracellular domain might be similarly important for keratinocyte cell-to-cell adhesion. We suppose that some pemphigus autoantibodies might bind to epitopes on these regions of DC I/II, which in turn might contribute to the loss of adhesion between keratinocytes. The direct evidence that this process does occur in pemphigus patients is lacking but it has been shown that Fab' fractions of an anti-DC antiserum inhibit desmosome assembly when added to the culture medium of subconfluent cells [33].

In conclusion, DC I/II are recognized by certain sera from patients with various types of pemphigus, but the significance of this reactivity remains to be defined.

Finally, this study shows that certain PV sera react with both desmoglein and the 135-kD PV antigen on immunoblots of bovine desmosome preparation, which confirms previous data [3]. Our results also indicate that despite the fact that BPF and PF sera are consistently bovine PV antigen-negative, some of these sera may react with the 130-kD PV antigen on immunoblots of human epidermal extracts. Nevertheless, there is a considerable difference of reactivity between PV and PF sera, which enables us to diagnose almost all pemphigus cases as PV or PF by immunoblotting. However, further immunoblot studies with the use of various antigen sources should be performed to unequivocally prove our previous suggestion [34] that the immunoblot technique can be used to distinguish PV and PF.

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