The Pathophysiological Significance of Nondesmoglein Targets of Pemphigus Autoimmunity

Development of Antibodies Against Keratinocyte Cholinergic Receptors in Patients With Pemphigus Vulgaris and Pemphigus Foliaceus

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Objectives: To determine whether nondesmoglein (non-Dsg) autoantibodies are pathogenic and whether they recognize keratinocyte cholinergic receptors that control cell adhesion because antikeratinocyte autoimmunity in patients with pemphigus vulgaris is not limited to the development of autoantibodies to Dsg.

Design: To determine whether non-Dsg autoantibodies are pathogenic, we sought to induce pemphigus in genetically engineered neonatal mice lacking Dsg 3 using pemphigus vulgaris IgGs that did not cross-react with Dsg 1. To determine whether pemphigus autoimmunity involves keratinocyte cholinergic receptors, the latter were separated from cell membranes of human keratinocytes, tagged with the covalent label \[3H\]propylbenzilylcholine mustard, and used as an antigen in a radioimmunoprecipitation assay of 34 pemphigus vulgaris and 6 pemphigus foliaceus serum samples.

Setting: The dermatologic clinics of the University of Minnesota, Minneapolis; the Mayo Clinic, Rochester, Minn; and the University of California–Davis Medical Center, Sacramento.

Patients: Serum samples were collected from 34 patients with pemphigus vulgaris and 6 patients with pemphigus foliaceus (aged 31-89 years) and from 7 age-similar patients of both sexes with nonpemphigus blistering or the following immune-mediated conditions: pemphigoid gestationis, bullous drug eruption, lupus erythematosus, erythema nodosum, urticaria, acute contact dermatitis, and skin ulcers.

Main Outcome Measures: Clinical, laboratory, and histopathologic findings.

Results: Extensive skin blistering accompanied by the Nikolsky sign and suprabasilar acantholysis was induced in the Dsg3null mice that received pemphigus, but not normal human IgGs. In the radioimmunoprecipitation assays for reactivity with cholinergic receptors, the mean radioactivity precipitated by pemphigus serum samples significantly exceeded both normal- and disease-control levels (\(P = .001-.02\)). The mean individual levels of radioactivity precipitated by 34 pemphigus vulgaris and pemphigus foliaceus serum samples (85%) exceeded control values by a mean of approximately 2.6 times.

Conclusions: Autoantibodies to keratinocyte cell-surface molecules other than Dsg 1 and Dsg 3 can induce clinical features of pemphigus vulgaris. Patients with pemphigus vulgaris and those with pemphigus foliaceus develop IgG antibodies that precipitate radiolabeled cholinergic receptors. Because these receptors control keratinocyte adhesion and motility, their inactivation by autoantibodies may elicit intracellular signals that cause disassembly of desmosomes, leading to acantholysis and blistering.

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Skin blisters occur in a variety of dermatologic conditions. Blisters in pemphigus are associated with the binding of IgG autoantibodies to keratinocyte cell-surface molecules. These “intercellular” or “pemphigus” antibodies bind to keratinocyte desmosomes and to desmosome-free areas of the keratinocyte cell membrane. Intercellular spaces enlarge, desmosomes decrease in number and eventually disappear, and the cells round up and detach from one another without cell death. This phenomenon is called “acantholysis.” Use of high doses of glucocorticosteroids (prednisone, sometimes up to 1000 mg/d) is required to suppress acantholysis. Corticosteroids are the mainstream of pemphigus management, but use of these drugs can cause severe adverse effects and complications, including death. The development of non-hormonal treatment of patients with pemphigus is pending elucidation of the pathophysiological pathways mediating acantholysis.

Some IgG antibodies found in serum from patients with pemphigus bind...
PATIENTS, MATERIALS, AND METHODS

CHARACTERIZATION OF PEMPHIGUS AND CONTROL SERUM SAMPLES

Serum samples were collected from 17 men and 23 women with pemphigus (aged 31-89 years). The study was approved by the local human subjects committees. Thirty-four patients had pemphigus vulgaris and 6 had pemphigus foliaceus. The presence of pemphigus antibodies and the antibody titers were determined by indirect immunofluorescence (IIF) using monkey esophagus as substrate. The serum samples were also applied to the experimental (Dsg 3–negative) and control (Dsg 3–positive) mouse skin specimens. The 6-µm-thick slices of the Dsg experimental (Dsg 3–negative) and control (Dsg 3–positive) mouse skin specimens. The 6-µm-thick slices of the Dsg mouse skin specimens. The 6-µm-thick slices of the Dsg 3–negative and Dsg 3–positive skin specimens had been subjected to identical freezing, fixation, storage, and sectioning and were positioned side by side on the same slide to allow simultaneous treatment with primary and secondary antibodies. A semiquantitative IIF assay25 using a computer-assisted image analysis software package (Signal Analytics, Vienna, Va) was used to compare the relative amounts of pemphigus IgGs bound to keratinocytes in the epidermis of mice lacking and containing Dsg 3. This assay is based on calculating the intensity of fluorescence pixel by pixel by dividing the summation of pixels of fluorescence intensity by the area occupied by the pixels (ie, segment) and then subtracting the mean intensity of fluorescence of a tissue-free segment (ie, background). For each skin specimen, a minimum of 3 different segments in at least 3 different microscopic fields were analyzed, and the results were compared. The presence of Dsg 3 in the skin specimens was determined using rabbit anti-Dsg 3 antibody (Srotec, Raleigh, NC).

Disease-control serum samples were obtained from 7 age-matched within the 5 expected products (expected product size is 280 bp), matched within the sequence of the neonycin resistance gene of the plasmid pPN used to engineer the Dsg3null mouse.13 The polymerase chain reactions were carried out as described previously,41 including preheating at 94°C for 4 minutes; hot start with tag DNA polymerase (Promega, Madison, Wis); then 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes.

ISOLATION OF KERATINOCYTE CHOLINERGIC RECEPTORS

Keratinocyte cholinergic receptors were prepared from fresh human neonatal foreskins by incubating 3 × 5-mm pieces of minced foreskin for 90 minutes at 37°C and 5% carbon dioxide in RPMI 1640 medium (Sigma Chemical Co) supplemented with EDTA, 20 mmol. The epidermis was then scraped with a blade from underlying dermis, placed in ice-cold buffer A consisting of sodium phosphate buffer, 50 mmol (pH 7.4), supplemented with the following protease inhibitors: phenylmethylsulphonyl fluoride, 0.5 mmol; iodoacetamide, 1 mmol; benzamide, 1 mmol; bacitracin, 0.1 mg/mL; leupeptin, 4 µg/mL; soybean trypsin inhibitor, 5 µg/mL; peptatin A, 1 µg/mL; EDTA, 1 mmol; phenanthrolin, 0.5 mmol; and aprotinin, 1 µg/mL; and EDTA. 20 mmol. The epidermis was then scraped with a blade from underlying dermis, placed in ice-cold buffer A consisting of sodium phosphate buffer, 50 mmol (pH 7.4), supplemented with the following protease inhibitors: phenylmethylsulphonyl fluoride, 0.5 mmol; iodoacetamide, 1 mmol; benzamide, 1 mmol; bacitracin, 0.1 mg/mL; leupeptin, 4 µg/mL; soybean trypsin inhibitor, 5 µg/mL; peptatin A, 1 µg/mL; EDTA, 1 mmol; phenanthrolin, 0.5 mmol; and aprotinin, 1 µg/mL. The IgG fractions that did not cross-react with Dsg 1 were isolated from pemphigus vulgaris and normal-control serum samples by precipitation with 33% ammonium sulfate followed by dialysis in phosphate-buffered saline solution, pooled and injected intraperitoneally through a 30-gauge needle at a dose of 20 mg per 1 g of body weight into 10- to 12-hour-old progeny from 5 different litters that resulted from breeding 3 heterozygous female mice and a homozygous male (129 × BL/6)-Dsg3null mouse.

GENOTYPING

Among neonates with antibody-induced pemphigus, the homozygous Dsg3null mice lacking Dsg 3 were identified using polymerase chain reaction amplification of the sequences of genomic DNA extracted from tails of mice using a tissue kit (QIAamp, Qiagen, Santa Clarita, Calif). The polymerase chain reaction used 2 different sets of primers to amplify 2 different products from the wild-type Dsg3 gene. The first pair of Dsg 3 primers included the sense primer 5'-GGAGGAAACAGACTAACAGGC-3' and the antisense primer 5'-ACCATCAGAGGCCCAGAGA-3', matching the sequence located upstream of the ATG starting codon of the Dsg3 gene, and the antisense primer 5'-TCCTCTGCCCCCTCTTAGGT-3', matching the position of the antisense primer of the first pair of primers, and the antisense primer 5'-CTCCCAACTCTGCTAGAGT-3', matching within the 3' end portion of the first exon (expected product size is 200 base pairs [bp]). The second pair of Dsg 3 primers included the sense primer 5'-AGGTGAGATGACAGGAGATC-3' and the antisense primer 5'-CCCTTGGGGAGACAGGATG-3', (expected product size is 280 bp), matched within the sequence of the neomycin resistance gene of the plasmid pPN used to engineer the Dsg3null mouse.13 The polymerase chain reactions were carried out as described previously,41 including preheating at 94°C for 4 minutes; hot start with tag DNA polymerase (Promega, Madison, Wis); then 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 3 minutes.

PASSIVE TRANSFER OF PEMPHIGUS ANTIBODIES TO NEONATAL MICE LACKING Dsg 3

The IgG fractions that did not cross-react with Dsg 1 were isolated from pemphigus vulgaris and normal-control serum samples by precipitation with 33% ammonium sulfate followed by dialysis in phosphate-buffered saline solution, pooled and injected intraperitoneally through a 30-gauge needle at a dose of 20 mg per 1 g of body weight into 10- to 12-hour-old progeny from 5 different litters that resulted from breeding 3 heterozygous female mice and a homozygous male (129 × BL/6)-Dsg3null mouse.
and degrade acetylcholine. In epidermis, the autoantibody complex. To covalently radiolabel keratinocyte receptors expressed by keratinocytes accumulate within keratinocyte desmosomal cadherins, desmoglein (Dsg) 1 and Dsg 3. These cadherinlike molecules hold keratinocytes together. Because keratinocytes in pemphigus undergo acantholysis, a direct causal relationship of anti-Dsg antibody and pemphigus acantholysis seemed certain. However, neither transgenic mice with truncated Dsg 3 nor Dsg 3–knockout (i.e., Dsg3<sup>–/–</sup>) mice spontaneously develop visible skin blisters, implicating the involvement of novel keratinocyte cell-surface molecules and linked pathophysiological pathways. Indeed, IgGs from pemphigus serum samples and the antibody to Dsg 3 protein. In the receptor preparations, pemphigus antibodies recognized 2 major bands at 40 and 60 kD and several smaller proteins (data not shown).

**RADIOIMMUNOPRECIPITATION ASSAY**

To detect antibodies against keratinocyte cholinergic receptors in test serum, we designed a specific RIPA in which covalently radiolabeled cholinergic receptors were used as antigen, test serum samples were used as the source of the primary anticholinergic receptor antibody, and goat antihuman IgG Fc-fragment antibodies (Sigma Chemical Co) were used as the secondary antibodies to precipitate the antigen–primary antibody complex. To covalently radiolabel keratinocyte cholinergic receptors, 200 µL of receptor protein solution was preincubated with [<sup>3H</sup>]PrBCM, 5 nmol (15 mCi/mmol of the custom-made [<sup>3H</sup>]PrBCM; NEN), for 30 minutes at 37°C, after which the nombound [<sup>3H</sup>]PrBCM was filtered by centrifugation on the Centriprep Concentrators (Amicon Inc, Beverly, Mass). Before labeling, [<sup>3H</sup>]PrBCM was cyclized in phosphate-buffered saline solution, 10 mmol, at 30°C for 20 minutes to activate the aziridinum ions. The specificity of [<sup>3H</sup>]PrBCM binding was confirmed by abolishing the radioligand binding by preincubating solubilized receptors with nonradioactive PrBCM (NEN). For each RIPA, the receptors were first labeled with [<sup>3H</sup>]PrBCM; then resuspended in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free phosphate-buffered saline solution, 100 µL; mixed with the test serum prediluted with this buffer, 50 µL, or plain buffer alone to obtain blank values; and placed on ice. After 1 hour of incubation, the secondary antibody was added and the incubation was continued for 30 minutes, after which the immune complexes were precipitated, and radioactivity was measured in an LKB liquid scintillation counter.

Each serum sample was tested in triplicate (or quadruplicate) on at least 2 occasions and at 3 dilutions (i.e., 1:60, 1:90, and 1:120). To measure net radioactivity of immune complexes, the blank values were subtracted from total counts, and results were expressed as mean ± SD counts per minute. To standardize RIPA results, the radioactivity precipitated by each pemphigus serum sample was also expressed relative to its reference control value. To obtain the reference control value, every RIPA tested the pemphigus samples and at least 1 reference sample that was a randomly selected disease-control serum sample drawn at approximately the same time and kept frozen under the same conditions as the pemphigus serum samples tested in the given RIPA. Significance limits were determined using the Student’s t test.

**Acetylcholine** regulates adhesion and motility of human epidermal keratinocytes, and activation of cholinergic receptors can prevent, stop, and reverse acantholysis elicited by pemphigus antibodies in vitro. In the epidermis, acetylcholine acts as a cytokotransmitter subserving a biologically active, cytokineline cholinergic network. Human keratinocytes synthesize, store, release, and degrade acetylcholine. In epidermis, the auto- and paracrine-acting acetylcholine alters cellular functions by acting on keratinocyte cholinergic receptors of muscularic and nicotinic classes. Cholinergic receptors expressed by keratinocytes accumulate within regions of the cell membrane associated with the sites of cell-cell attachment (ie, at desmosomes), precisely where pemphigus IgG accumulates, as visualized by immunoelectron microscopy. The location of these receptors suggests their involvement in regulating cell-cell attachments mediated by cadherin molecules. Inactivating keratinocyte cholinergic receptors using muscarinic (atropine sulfate) or nicotinic (mecamylamine hydrochloride) antagonists leads to reversible cell-cell detachment and acantholysis in keratinocyte monolayers.

Patients with pemphigus occasionally develop myasthenia gravis. This autoimmune disease of neuromuscular transmission is caused by antibodies to cholinergic receptors expressed by muscle cells. Coexistent pemphigus and myasthenia gravis often develop in patients with thymoma, and the autoantibodies are directed toward epithelial, muscle, and thymic antigens. Autoantibodies causing neuromuscular block in patients with myasthenia gravis recognize cholinergic receptors expressed on the cell surface of thymic epithelial cells. These cells can be stained specifically with pemphigus IgGs. Therefore, cholinergic receptor(s) expressed by epidermal keratinocytes might share common antigenic epitope(s) with muscle and thymic cholinergic receptors. Results of pilot studies by Grando et al demonstrate that pretreatment of normal human epidermis with serum from a patient with coexistent pemphigus, myasthenia, and benign lymphocytic thymoma prevents staining of keratinocytes with rabbit antibody, recognizing a unique sequence region present in the
carboxyterminal of the α3 subunit of the keratinocyte cholinergic receptor. The IgGs from this patient also precipitated a radiolabeled cholinergic receptor solubilized from keratinocyte membranes.

Results of the present study demonstrate that (1) non–Dsg 1 and non–Dsg 3 serum antibodies from patients with pemphigus vulgaris are pathogenic because they induce suprabasilar acantholysis and extensive skin blistering in neonatal mice and (2) some of these non–Dsg 1 and non–Dsg 3 antibodies can be directed against keratinocyte cholinergic receptors because, as measured in the radioimmunoprecipitation assay (RIPA), pemphigus vulgaris and pemphigus foliaceus IgGs precipitate cholinergic receptors solubilized from human keratinocytes and covalently labeled with the cholinergic radioligand [3H]propylbenezylcholine mustard ([3H]PrBCM). These results suggest that acantholysis in patients with pemphigus is mediated through an autoantibody-induced inactivation of keratinocyte cholinergic receptor(s), disrupting the physiologic control of cell adhesion.

**RESULTS**

**BINDING OF PEMPHIGUS ANTIBODIES TO KERATINOCYTES IN THE EPIDERMIS OF MICE LACKING Dsg 3**

To test the hypothesis that antikeratinocyte autoimmunity in pemphigus vulgaris is not limited to antibodies against Dsg 3, we performed IIF experiments testing the ability of patients’ serum to bind to keratinocytes in the epidermis of (129 × BL/6)-Dsg3null mice, which lack Dsg 3. All pemphigus vulgaris serum samples stained both interfollicular (Figure 1, A) and follicular (Figure 1, B) areas of epidermis in a fishnetlike, pemphigus pattern consistent with binding of non–Dsg 3 pemphigus antibodies to keratinocyte membranes. When the intensities of fluorescence of Dsg 3–free (ie, Dsg3null) and Dsg 3–containing (ie, Dsg3+/+) specimens were compared by a semiquantitative IIF assay, the epidermis of Dsg3null mice immunoreacted with approximately 58% of the relative amounts of pemphigus IgGs bound in the epidermis of the Dsg3+/+ BALB/c mice, defined as 100%.

Because some patients with generalized pemphigus vulgaris may develop antibodies to Dsg 1,53 we examined the immunospecificities of the pemphigus vulgaris serum samples that stained the epidermis of Dsg3null mice. It was found that the immunospecificities of the pemphigus vulgaris serum samples was limited to epidermal proteins with apparent molecular masses of 40, 60, and 70 kD, in addition to the 130-kD Dsg 3 band, and specifically excluded the Dsg 1 band of 160 kD (Figure 2, lanes 1-3). Epidermal extract used for immunoblotting included the intact Dsg 1 antigen because it was visualized by antibodies from pemphigus foliaceus serum (Figure 2, lane 4). These pemphigus vulgaris serum also specifically stained keratinocyte membranes in the epidermis of another genetic stock of Dsg 3–negative mice, namely, “balding” C57/6-Dsg3null/Dsg3null mutant mice (data not shown). These mice have a spontaneous recessive mutation within the Dsg3 locus on mouse chromosome 1813 and do not express Dsg 3.13

The absence of Dsg 3 from Dsg3null and Dsg3null/Dsg3null epidermal specimens was confirmed by their negative staining with an antibody to Dsg 3 protein that stained keratinocytes in the epidermis of control BALB/c, C57Bl6+/−, and (129 × BL/6) + /Dsg3null mice equally efficiently (data not shown).

**PATHOGENICITY OF NON–Dsg 1 AND NON–Dsg 3 ANTIBODIES FROM PEMPHIGUS VULGARIS SERUM**

The observation that serum autoantibodies from patients with pemphigus vulgaris stain epidermis of mice lacking Dsg 3 in the pemphigus-specific pattern led us to determine whether these non–Dsg 1 and non–Dsg 3 antibodies are pathogenic. Because neonatal Dsg3null mice do not show any evidence of spontaneous acantholysis and blistering,13 these pups were chosen as the animal model to further study the pathophysiological significance of non–Dsg 1 and non–Dsg 3 antibodies produced by patients with pemphigus vulgaris.

The intraperitoneal injection of pooled pemphigus vulgaris IgG fractions that did not cross-react with Dsg 1 (Figure 2, lanes 1-3), but not normal human IgGs, into 1-day-old progeny of Dsg3null mice or control Dsg3+/+...
BALB/c mice produced gross skin blistering approximately 18 to 24 hours after injection in both groups. There were no differences in the time of onset or in the extent of blister formation between these 2 breeds that differed in Dsg3 expression. At the onset of skin blistering, the following 3 variants of the Nikolsky sign, representing a loss of peripheral cohesion, were elicited: (1) peripheral enlargement of an intact blister secondary to vertical pressure on its roof, (2) linear extension of the margin of the existing erosion by pulling the remnant of blister roof with tweezers, and (3) epidermal split of clinically noninvolved skin by applying lateral traction with a pencil eraser. When blisters became generalized, the mice were killed, and the homozygous Dsg3null pups were identified by genotyping. Visualization of 2 polymerase chain reaction products of 200 and 500 bp identified the homozygous Dsg3null mouse, and 3 products of 200, 500, and 280 bp indicated the heterozygous Dsg3+/− mouse, 3 products of 200, 300, and 280 bp identified the heterozygote Dsg3+/− mouse, and 1 product of 280 bp represented the homozygous Dsg3−/−, or Dsg3null, mouse (Figure 3).

The gross and microscopic changes of the skin of a Dsg3null mouse injected with pemphigus IgGs are shown in Figure 4. All littermates were normal at birth (injection of healthy IgGs did not induce any gross or microscopic changes of the skin of Dsg3null mice or their littermates; Figure 4, A). By 18 hours after injection, a flaccid skin blister with serous exudate spontaneously developed on the abdomen of a Dsg3null mouse (Figure 4, B). At this time, the Nikolsky sign was elicited (Figure 4, C).

Light microscopic examination of the marginal blistered areas revealed intraepidermal pathologic changes with suprabasilar acantholysis and basilar “tombstoning” characteristic of pemphigus vulgaris (Figure 4, D). Early stages of keratinocyte cell-cell detachment were observed in biopsy specimens of clinically noninvolved skin (Figure 4, E). This likely accounts for the positive Nikolsky sign on intact skin. Direct immunofluorescence of both lesional and clinically normal skin showed pemphigus IgG localized to keratinocyte cell membranes, producing the classic fishnetlike, pemphigus staining pattern (Figure 4, F).

**REACTION OF PEMPHIGUS ANTIBODIES WITH KERATINOCYTE CHOLINERGIC RECEPTORS**

Pemphigus serum samples were found to specifically react with keratinocyte cholinergic receptors. The mean absolute values of radioactivity precipitated by pemphigus serum samples always significantly (P<.05) exceeded both the disease-control and the normal-control values (Figure 5). Radioactivity precipitated by disease-control serum samples in RIPA did not significantly differ from that precipitated by normal human serum samples (Figure 5).

Analysis of standardized results comparing individual pemphigus serum samples (Figure 6) revealed that 34 (85%) of 40 pemphigus serum samples precipitated more radioactivity than their reference control values. The mean radioactivity precipitated by these 34 pemphigus serum samples exceeded their reference control values by a mean of 2.6 times (range, 1.1-7.8).

The clinical form of pemphigus did not affect the ability of serum to precipitate cholinergic receptors. Pemphigus vulgaris and pemphigus foliaceus serum samples always precipitated more radioactivity than their reference control values.

**COMMENT**

The pathophysiology of pemphigus vulgaris is not limited to the autoimmune against the keratinocyte adhe-
sion molecule Dsg 1 or Dsg 3 because patients' serum IgGs that do not recognize Dsg 1 induce pemphigus in genetically engineered mice lacking Dsg 3. Patients with pemphigus vulgaris and pemphigus foliaceus develop antibodies to cholinergic receptors expressed on the cell surface of human epidermal keratinocytes. These anticholinergic receptor antibodies may contribute to the development of acantholysis and blister formation in pemphigus by disrupting the signals from keratinocyte cholinergic receptors that activate adhesion molecules.

We studied non-Dsg antibodies in pemphigus because of the failure to identify Dsg 3 as an exclusive target molecule responsible for the clinical manifestations of pemphigus. The following observations suggest that keratinocyte cell-surface molecules other than Dsg 3 are responsible for blister formation in patients with pemphigus vulgaris:

Figure 4. Induction of pemphigus in neonatal mice lacking desmoglein (Dsg) 3 by passive transfer of IgGs from pooled serum of 3 patients with pemphigus vulgaris. A, Four-day-old progeny from mating of a heterozygous Dsg3+/− female and a homozygous male Dsg3null mouse after a course of 3 intraperitoneal injections of normal IgGs at a dose of 20 mg per 1 g of body weight per day. All littermates appear similar because there are no spontaneous or normal IgG-induced skin changes in pups lacking Dsg 3. B, Large, flaccid blister filled with serous fluid that developed on the skin of a Dsg3null mouse approximately 18 hours after a single intraperitoneal injection of pemphigus vulgaris IgGs at a dose of 20 mg per 1 g of body weight. C, Positive Nikolsky sign. Mechanical extension of a large erosion on the abdominal skin of the Dsg3null mouse after spontaneous rupture of the blister. D, Intraepidermal separation at the edge of the skin erosion of the Dsg3null mouse showing acantholysis and prominent tombstone appearance of the basal cell layer (hematoxylin-eosin, original magnification ×200). E, Suprabasilar cleft in the clinically intact skin on the back of the same Dsg3null mouse (hematoxylin-eosin, original magnification ×630). F, Direct immunofluorescence of the perilesional skin of the Dsg3null mouse with fluorescein isothiocyanate–conjugated antihuman IgG antibody localizing pemphigus IgGs to keratinocyte membranes (original magnification ×1000).
1. Intercellular autoantibodies recognizing the 130-kd epidermal protein (Dsg 3) are present at significant titer in the serum of approximately half of the first-degree relatives of patients with pemphigus vulgaris but fail to cause any skin or mucous membrane changes.

2. Neonatal mice injected with pemphigus vulgaris IgG purified by a fusion protein representing the “pathogenic” extracellular domains of Dsg 3, i.e., EC1 and EC2, show pemphigus-like epidermal staining and signs of cell-cell detachments visible on microscopy but do not develop gross skin blistering despite an appreciable titer of antibody in mouse serum.

3. Transgenic mice with amino terminal deletion of Dsg 3 show swelling of paws and digits, focal frankness of the skin, and necrotic changes on the tips of the tails but no loss of cell-cell adhesion and no evidence of skin blisters.

4. The homozygous balding Dsg3null/Dsg3null mice harboring a spontaneous recessive mutation that inactivates Dsg3 gene expression show alopecia and mucous membrane vesicles and erosions but only rare interfollicular epidermal changes and no visible blisters.

5. Dsg3null mice with a targeted null mutation of the Dsg3 gene are healthy at birth but develop runting and hair loss around the time of weaning and may exhibit crustled lesions around the eyes, on the snout, and on the nipples of nursing mothers, apparently from trauma. These mice, however, do not develop spontaneous skin blisters or extensive erosions.

In addition to the 130-kd Dsg 3 and 85-kd plakoglobin complex, patients with pemphigus may develop autoantibodies to keratinocyte proteins with apparent molecular masses of 12, 18, 47, 50, 52, 55, 59, 66, 67, 75, 78, 102, 105, 180, and 190 kd. Rabbit antibody raised against the 66-kd protein reproduced pemphigus in 4 of 5 injected mice. The pemphigus vulgaris serum samples used in the present study to induce pemphigus in neonatal Dsg3null mice also recognized keratinocyte proteins with apparent molecular masses of 40, 60, and 70 kd. Binding of pemphigus IgGs to one of these yet unidentified keratinocyte antigens may mediate acantholysis and blistering. To rule out a possibility that conformational epitope of Dsg 1 was destroyed in the course of immunoblotting procedures, thus rendering false-negative results, the immunoreactivities of pemphigus vulgaris IgGs that can induce symptoms of pemphigus in neonatal Dsg3null mice were further characterized by a combination of immunoprecipitation techniques featuring various degrees of sensitivity and specificity (V.T.N. and S.A.G., unpublished data, 1998). The absence of immunoreactivity of these serum samples with the 160-kd Dsg 1 antigen precludes the possibility that the serum targeted this desmosomal cadherin to cause acantholysis.

The fate of pemphigus antibodies subsequent to binding to the cell surface of cultured murine keratinocytes suggests that pemphigus antibody interacts with its target in a receptor/ligand manner, eliciting an intracellular signal that translates into abnormalities in adhesive function of the cells. Accordingly, binding of pemphigus IgGs to cultured keratinocytes causes activation of phospholipase C, production of inositol 1,4,5-trisphosphate, Ca2+ influx and rapid transient increase of intracellular Ca2+, changes in the intracellular cyclic adenosine monophosphate–cyclic guanosine monophosphate ratios, and activation and translocation of protein kinase C from the cytosol to the particulate and/or cytoskeleton fractions. The expression and/or function of the proteins mediating intercellular junctions of keratinocytes is also regulated by the protein kinase C–mediated pathway, and binding of pemphigus antibodies to cultured keratinocytes leads to phosphorylation of desmosomal cadherins. Therefore, it is reasonable to speculate that binding of pemphigus antibody to keratinocyte cell-surface receptor(s) initiates an intracellular signaling cascade that leads to disassembly of desmosomes.
We believe the cholinergic receptor on the cell surface of keratinocytes is a target for pemphigus antibodies because (1) myasthenia gravis, an anti-acetylcholine receptor autoimmune disease, is an uncommon but distinctive concomitant of pemphigus; (2) suramin sodium (germanine or naphuride), a drug that acts on an acetylcholine receptor, has been used to treat pemphigus (reviewed by Lever); and (3) acetylcholine, synthesized by human keratinocytes, regulates keratinocyte adhesion in an autocrine and paracrine manner and reverses pemphigus IgG–induced acantholysis in keratinocyte monolayers. Results of RIPAs indicate that patients with pemphigus often develop antibodies that bind keratinocyte cholinergic receptors. The absolute values of radioactivity precipitated by pemphigus serum significantly exceeded both disease-control and normal-control values. The cholinergic receptors were isolated from keratinocyte plasma membranes using a standard procedure of cholinergic receptor solubilization in a mixture of digitonin and sodium cholate. The fact that the receptor preparation contained functional cholinergic receptors was demonstrated by saturable specific binding ($B_{\text{max}} = 0.9$ nmol/0.175 mg protein) of $[^3H]$N-methylscopolamine, a cholinergic drug that specifically binds to all known subtypes of the muscarinic class of cholinergic receptors. $[^3H]$propylbenzilycholine is commonly used to selectively label cholinergic receptors in the receptor preparations and to visualize solubilized receptor proteins using autoradiography of electrophoresis gels. When $[^3H]$PrBCM-labeled keratinocyte cholinergic receptors were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, 2 radioactivity peaks were found, a major one at approximately 60 kd and a minor one at approximately 95 kd. These proteins migrated with keratinocyte muscarinic receptors, as judged from their visualization on immunoblotting membranes treated with the monoclonal antibody M35, which recognizes all 5 known muscarinic cholinergic receptor subtypes. In this study, the specificity of $[^3H]$PrBCM binding to the receptors was confirmed by competitive inhibition of radioligand binding to solubilized receptors by nonradioactive PrBCM. The fact that this drug binds to the receptor molecules irreversibly, via an alkylating reaction, precluded the use of $[^3H]$PrBCM-labeled receptors in the binding inhibition experiments using pemphigus IgGs as a competitor. However, in experiments with the reversible radioligand $[^3H]$atropine, Grando and Dahl previously demonstrated that preincubation of keratinocytes with pemphigus, but not normal, IgGs significantly diminishes
nocyte membranes.
Propylbenzilycholine, like atropine, specifically binds to the muscarinic class of cholinergic receptors, and a muscarinic receptor has been implicated in mediating control of cell adhesion by the cholinergic drug carbachol via a protein kinase C-mediated pathway that activates E-cadherin. We therefore speculate that pemphigus antibodies target one of several molecular subtypes of muscarinic cholinergic receptors expressed on the surface of human keratinocytes. However, it also has been shown that PrBCM can label nonmuscarinic receptors, such as amiloride-sensitive Na+/H+-exchanger. Pemphigus IgGs may precipitate other keratinocyte cholinergic molecules binding [3H]PrBCM, including members of the keratinocyte nicotinic cholinergic receptor family or a putative amiloride-sensitive Na+-channel with cholinergic properties. Furthermore, recent findings in our laboratory indicate that [3H]PrBCM labels a novel keratinocyte annexinlike molecule that was identified by pemphigus vulgaris IgGs in human keratinocyte Agt11 cDNA library.

We propose that acantholysis in pemphigus is mediated by at least 2 complementary pathogenic pathways, namely, (1) an anticholinergic receptor antibody that weakens intercellular adhesions between keratinocytes via inactivation of the cholinergic receptor-mediated physiologic control of cadherin (Dsg) expression and/or function and causes dyshesion, cell detachment and rounding up, or acantholysis and (2) antibodies to adhesion molecules that prevent formation of new desmosomes in acantholytic keratinocytes by blocking the extracellular domains of desmosomal cadherins that mediate homophilic adhesion.

These separate pathophysiological pathways targeting 2 different types of keratinocyte cell-surface molecules (ie, cholinergic receptor and Dsg) may explain the low- and high-affinity binding of pemphigus IgGs to keratinocytes. The present results indicate that anti-Dsg 3 antibodies represent approximately 40% and that non-Dsg 3 antibodies represent approximately 60% of the total antikeratinocyte autoantibodies circulating in the serum of patients with pemphigus vulgaris who do not develop anti-Dsg 1 antibodies. The existence of at least 2 types of antikeratinocyte antibodies in pemphigus may reconcile the failure of recombinant proteins representing different portions of Dsg 3 to pre-absorb from pemphigus serum the antibodies that can induce pemphigus in neonatal mice.

Complete absorption of disease-causing antibodies has been achieved using a chimeric protein representing a combination of Dsg 3 with the constant region of human IgG1. Complete absorption of disease-causing antibodies has been achieved using a chimeric protein representing a combination of Dsg 3 with the constant region of human IgG1. The combination of Dsg 3 and IgG1 peptide region may create a conformational epitope that mimics pathogenic pemphigus vulgaris antigen. Because pemphigus vulgaris IgGs that do not cross-react with Dsg 1 reproduce pemphigus in Dsg3null mice lacking Dsg 3, anti-Dsg 3 antibody and Dsg 3 itself unlikely play critical roles in the pathophysiological development of pemphigus. Perhaps the folding of the chimeric protein simulates some other keratinocyte protein, such as cholinergic receptor. Alternatively, the disease-causing antibodies may have been eliminated through their affinity to the IgG1 portion of the chimeric protein. It recently has been proposed that selected autoantibodies produced during the course of an autoimmune disease behave as adhesion molecules via molecular mimicry of the motif involved in protein-protein adhesion.

In conclusion, the results of this study provide the first direct evidence that the antibodies to the keratinocyte cell surface molecules other than Dsg 1 and Dsg 3, which are produced by patients with pemphigus vulgaris, are indeed pathogenic. These novel, pathophysiologically important targets may include keratinocyte cholinergic receptor(s). The presence of antibodies against keratinocyte cholinergic receptors in pemphigus, together with the knowledge that cholinomimetic drugs can modulate pemphigus IgG-induced acantholysis in vitro, suggests that clinical trials of tissue- or receptor-class-specific cholinomimetics may be warranted in both pemphigus vulgaris and pemphigus foliaceus. Studies are in progress to identify the molecular structure of keratinocyte cholinergic receptor(s) precipitated by pemphigus serum and to determine other novel antigens recognized by pemphigus antibodies.

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