Pemphigus Vulgaris Antibody Identifies Pemphaxin

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Because pemphigus vulgaris (PV) IgGs adsorbed on the rDsg3-Ig-His baculoprotein induced blisters in neonatal mice, it was proposed that anti-desmoglein 3 (Dsg 3) autoantibody causes PV. However, we found that rDsg3-Ig-His absorbs autoantibodies to different antigens, including a non-Dsg 3 keratinocyte protein of 130 kDa. This prompted our search for novel targets of PV autoimmunity. The PV IgG eluted from a 75-kDa keratinocyte protein band and also contained antibodies to a pemphigus-like pattern and induced acantholysis in keratinocyte monolayers. Screening of a keratinocyte Agt11 cDNA library with this antibody identified clones carrying cDNA inserts encoding a novel molecule exhibiting ~40% similarity with annexin-2, named pemphaxin (PX). Recombinant PX (rPX-His) was produced in Escherichia coli M15 cells, and, because annexins can act as cholinergic receptors, its conformation was tested in a cholinergic radioligand binding assay. rPX-His specifically bound [3H]acetylcholine, suggesting that PX is one of the keratinocyte cholinergic receptors known to be targeted by disease-causing PV antibodies. Preabsorption of PV sera with rPX-His eliminated acantholytic activity, and eluted antibody immunoprecipitated native PX. This antibody alone did not cause skin blisters in vivo, but its addition to the preabsorbed PV IgG fraction restored acantholytic activity, indicating that acantholysis in PV results from synergistic action of antibodies to different keratinocyte self-antigens, including both acetylcholine receptors and desmosomal cadherins.

Pemphigus vulgaris (PV)† is a potentially lethal disease of skin adhesion in which keratinocytes (KC), the stratified epithelial cells comprising the epidermis, lose their ability to adhere to one another (acantholysis) (1). Acantholysis leads to an intra-epidermal split and separation of the suprabasal epidermal layer, which is clinically manifested by blistering that denudes skin and oral mucosa. Introduction of glucocorticosteroids into the treatment of PV patients decreased mortality from 90 to 10% (reviewed in Ref. 2). Long-term corticosteroid therapy of PV patients is life-saving but causes severe side effects, including death (3, 4). This urgent development of non-hormonal therapy of pemphigus acantholysis. The pathophysiology of PV includes an array of IgG autoantibodies reacting with keratinocyte self-antigens with the apparent molecular mass ranging from 12 to 190 kDa (reviewed in Ref. 5), including a 130-kDa keratinocyte polypeptide (6, 7). The notion that autoantibodies are the main cause of PV stems from the fact that passive transfer of pemphigus, but not normal, IgGs to neonatal mice can induce skin lesions characteristic of PV (8). Using pemphigus antibodies eluted from the 130-kDa band as a probe, Amagai et al. (9) screened the human keratinocyte Agt11 cDNA library and found that two of the clones recognized by these PV antibodies represented a novel desmosomal cadherin termed desmoglein (Dsg) 3. The hypothesis that PV, a disease of skin adhesion, is caused by an antibody to Dsg 3, an adhesion molecule, prompted experiments toward elucidation of the biological effects of anti-Dsg 3 antibody. However, acantholysis could not be documented in keratinocyte monolayers treated with anti-Dsg 3 antibody. Several recombinant Dsg 3 (rDsg3) proteins were produced and used to test if adsorbed antibodies can elicit skin blistering in neonatal mice upon passive transfer (10, 11). Although rDsg3 could absorb PV antibodies to Dsg 3, it failed to absorb all disease-causing antibody, and PV IgGs depleted of antibodies to Dsg 3 kept binding to KC in murine epidermis and inducing gross skin blisters (10, 12). Only creation of a chimeric baculoprotein that included both the extracellular epitope of Dsg 3 and an Fc portion of human IgG1 could fulfill both goals: elimination of all disease-causing antibodies from pemphigus serum and induction of gross skin blisters in neonatal mice injected with concentrated eluants (13, 14). Explanations of this phenomenon include: 1) a possibility that the IgG portion rendered the rDsg3 with appropriate conformational epitope, which could be tested by crystallography; and 2) a possibility that the tertiary structure of the chimera mimicked non-Dsg 3 targets of pemphigus autoimmunity, which could be tested by characterizing the antigenic profile of the eluted IgG. Neither possibility was tested. Recently, it has become evident that anti-Dsg 3 antibody alone is not sufficient to cause skin blisters (15). A role for an autoantibody to another desmosomal cadherin, Dsg1, was
Pemphaxin—New Annexin, Pemphigus Antigen, Cholinergic Receptor

proposed to explain skin blisters in PV patients (16). However, well-documented cases of generalized disease in PV patients lacking Dsg1 antibody (17) argued in favor of the existence of a yet unidentified disease-causing non-Dsg1/Dsg3 antibody that could have been nonspecifically preabsorbed with rDsg3-Ig constructs. Furthermore, intraperitoneal injection of the PV IgG, which did not have anti-Dsg1 activity, into neonatal Dsg3 knockout mice (i.e., Dsg3Null mice) resulted in gross skin blisters (5). It should be mentioned that neonatal Dsg3Null mice lack the true PV phenotype, in that they do not develop spontaneous skin blisters (5, 18), which has already justified their use in passive transfer experiments by different research groups studying the nature of disease-causing PV antibodies (5, 15).

Recently, we have compared antibodies eluted from rDsg3 (rDsg3-His) and rDsg3-Ig (rDsg3-Ig-His), which were used in the original preabsorption experiments (10, 13, 14), and demonstrated that the two Dsg 3 constructs adsorb antibodies with different antigenic specificities (19). The PV IgGs eluted from rDsg3-Ig-His reacted predominantly with the 130-kDa protein band present in normal human KC in addition to a few weakly stained bands that varied among test PV sera. In marked contrast, the antibodies eluted from rDsg3-Ig-His recognized different protein bands, including a non-Dsg 3 130-kDa band in the immunoblot of Dsg 3/–/– keratinocyte proteins. Thus, crossreactivity of Dsg3-Ig-His with non-Dsg 3 antibodies explains how this chimeric baculoprotein could absorb all disease-causing PV IgG.

The vast majority of pemphigus patients develop antibodies that immunoprecipitate keratinocyte membrane proteins binding the covalent cholinergic radioligand [3H]propylbenzilylcholine mustard ([3H]PBCM) (5) and compete with a cholinergic radioligand, [3H]labeled acetylcholine ([3H]ACh) receptor(s) targeted by PV autoimmunity remains to be determined. Addition to either muscarinic or nicotinic antagonists to keratinocyte monolayers in both cases results in acantholysis (reviewed in Refs. 21, 22), whereas cholinergic agonists stimulate cell-to-cell adhesion of KC, and can reverse, attenuate, or prevent acantholysis in keratinocyte monolayers when added to culture after, simultaneously with, or prior to PV IgG, respectively (20). The anti-acantholytic activity of cholinergic agonists suggests a novel avenue for development of non-hormonal treatment of pemphigus.

In this study, we demonstrate the nature of a novel target for non-Dsg 3 disease-causing PV IgG. Screening of the keratinocyte cDNA expression library with PV IgG immunoaffinity-purified on a 75-kDa area of the immunoblotting membrane revealed a novel human annexin-like molecule, which we named pemphaxin (PX). We produced recombinant PX (rPX-His) and demonstrated that this protein acts as a cholinergic receptor in the radioligand binding assay with [3H]ACh. PV IgG specifically recognized rPX-His, and preabsorption of PV sera with rPX-His eliminated the acantholytic activity that could be restored by adding back the anti-PX antibody eluted from the affinity column. Thus, disease-causing PV antibody identified PX, a novel human annexin that acts as a keratinocyte cell surface receptor for ACh, and, therefore, may mediate known biological effects of this cysotransmitter on adhesion and motility of KC.

**EXPERIMENTAL PROCEDURES**

*Sources of Sera and Tissue*—The sera and IgG fractions were from well-established PV patients, and from healthy volunteers. This study had been approved by the University of California Davis Human Subjects Review Committee. The diagnosis of PV was made based on the results of both comprehensive clinical and histological examinations together with immunological studies, which included direct immunofluorescence (DIP), indirect immunofluorescence (IF) on various epithelial substrates, immunoblotting, and immunoprecipitation, following standard protocols (23). The serum samples were stored frozen at −80 °C until use in experiments. The serum IgG fractions were isolated using 40% ammonium sulfate followed by dialysis with CaCl2- and MgCl2-free phosphate-buffered saline (PBS; Life Technologies, Inc., Gaithersburg, MD), lyophilized, and reconstituted in PBS as detailed elsewhere (5). The protein concentration was determined using the Micro BCA kit (Pierce). The samples of normal human neonatal foreskins that were used to start keratinocyte cell cultures were transported to the laboratory in culture medium, and the samples of normal human abdominoplasty skin that served as a source of keratinocyte membrane protein for immunoblotting were frozen immediately after harvesting.

**Immunofinity Purification of Acantholytic Anti-keratinocyte PV Antibody**—The enriched fraction of human keratinocyte membrane protein (5) was used as a substrate in immunoblotting experiments aimed at characterizing novel PV antigens. The epidermis was separated from the dermis by incubation in RPMI 1640 medium (Sigma), supplemented to contain 200 mM EDTA for 90 min at 37 °C and 5% CO2 (24), and harvested into a 50-mL polyethylene centrifuge tube filled with ice-cold Tris-buffered saline (TBS), pH 7.4, that contained the following protease inhibitors: 2 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml bacitracin, 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml pepstatin A, and 10 μg/ml chymostatin (all from Sigma). The epidermis was washed three times with TBS, centrifugation put at −4 °C, and lyophilized. It is characterized by a 130-kDa band present in a PowerGen tissue-and-cell disrupter (Fisher Scientific, Santa Clara, CA) in the same buffer containing 20 mM Ca2+. Large organelles and epidermal debris were removed by centrifugation at 2000 × g for 45 min at 4 °C, and the cell membrane fraction was pelleted from the supernatant by centrifugation at 80,000 × g for 1 h at 4 °C. The pellet was solubilized in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer containing 2% SDS and 5% β-mercaptoethanol, boiled for 5 min, and cleared by centrifugation at 40,000 × g for 1 h at 4 °C. Western blotting of SDS-PAGE-resolved proteins was performed as reported previously (5) with minor modifications. Briefly, the proteins were separated on a 7.5% SDS-PAGE gel and transferred to an Immobilon-P membrane (Millipore Corp., Bedford, MA), which was blocked, first with 5% milk in TBS for 1 h at 37 °C and then with TBS containing 1% normal goat serum, 3% dried milk, and 0.05% Tween 20 (Sigma) overnight at 4 °C, and cut into 4-mm wide vertical strips. Each strip was exposed to a primary antibody, i.e., PV or normal human serum, for 1 h at room temperature and then washed thoroughly. The protein bands recognized by PV and normal human IgG were visualized using goat anti-human IgG antibody (Hybridoma Bank, University of Iowa, Iowa City, IA) which was developed using a biotin-streptavidin ABC system (Vector Laboratories, Burlingame, CA). The specificity of binding was determined in negative control experiments, in which the primary antibodies were omitted. The PV IgG fractions were isolated from the immunoblotting membrane areas that were recognized uniquely by PV IgG, but not normal human IgG, following a procedure described previously (25). Briefly, approximately 3-mm wide horizontal strips carrying a keratinocyte membrane protein with a particular molecular mass of ≥3 kDa were cut out from the immunoblotting membrane and incubated overnight with PV serum diluted 1:5 in TBS containing 20 mM CaCl2, 0.05% Tween 20 (Sigma), and 1% non-fat skim milk to allow antibody binding. The strips were then washed thoroughly, and the antibodies were eluted by a 3-min incubation at 37 °C in a solution containing 500 μl of 20 mM sodium citrate, 1% milk, and 0.05% Tween 20 (pH 3.2) and immediately neutralized by adjusting the pH to 7.4 with the 2 M Tris base.

**Immunofluorescence Screening Experiments**—The IIF experiments testing the ability of PV IgG eluted from the strips of immunoblotting membranes to specifically stain KC in the tissue samples were performed as described previously (5) with minor modifications. Briefly, 4- to 8-mm cryostat sections of freshly frozen normal human skin, monkey esophagus, or murine skin were incubated overnight at 4 °C with the immunoaffinity-purified PV IgG fractions, after which the tissue sections were washed and binding of primary antibody was visualized by incubating the tissue section with fluorescein isothiocyanate (FITC)-labeled goat anti-human IgG antibody (Fierce) for 1 h at room temperature. The specificity of antibody binding was demonstrated by omitting the primary antibody, which abolished the staining. The immunofluorescence images were obtained using a fluorescence microscope (Axiostar, Carl Zeiss Inc., Thornwood, NY) with a charge-coupled device video camera (Photon Technology International, Monmouth Junction, NJ) attached.

**Cell Culture Screening Experiments**—Acantholytic activity of the eluted PV IgGs, which stained the stratified epithelial substrate in a...
pephimmic-like, “intercellular” pattern, were tested in the monolayers of normal human foreskin KC isolated from the epidermis and grown at 37 °C in serum-free keratinocyte growth medium (KGM; Life Technologies, Inc.) containing 0.09 mM Ca2+ in a humid 5% CO2 incubator, as detailed elsewhere (28). To observe changes in cell morphology, second passage KC were seeded into 6-well plates and treated with 8-% buffered formalin. The cells were neutralized, blocked with 3% non-fat milk in TBS and screened for colonies that produced the rPX-His protein. The transformed bacterial colonies were blotted to a nitrocellulose filter and inversely placed on an IPTG-containing NYZ agar plate and grown overnight at 37 °C. Over 3 million plaques formed on the bacterial plate. The size of the PCR product was confirmed by gel electrophoresis and Coomassie Blue staining. The correct PX cDNA had proper frame and orientation. A representative clone was isolated. The insert from isolated clones were amplified with a pair of cloning primers specific for the Sph I and Kpn I sites in the multiple cloning site of the pQE-30 Sph I-gt11 vector: 5′-ggtagcgaccg-cagac-3′ (forward) and 5′-ggtagcgaccg-tgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtggtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtg-
from the column by Immunopure Gentle elution buffer and desalted on a v-Salt Exelulose plastic desalting column (both from Pierce). The pattern of specific binding of the eluted antibody was examined by IIF on human skin and monkey esophagus. The antigenic profile of the eluted PV IgG was identified by immunoprecipitation of metabolically labeled human keratinocyte proteins (see below), which is considered the most sensitive and specific approach to characterize the antigenic specificity pemphigus antibodies (7).

Metabolic Labeling of Cultured KC and Immunoprecipitation Assay—Second passage human foreskin KC were grown to approximately 90% confluence, washed thoroughly with prewarmed (37°C) PBS, incubated for 15 min at 37°C in methionine-free Dulbeco's modified Eagle's medium (Life Technologies, Inc.) containing 15% newborn calf serum, and then exposed to 100 μCi/ml [35S]methionine (1000 Ci/mmol, Amershams Pharmacim Biotech, Arlington Heights, IL) in 1.8 mM Ca2+ labeling medium for 16 h in a humid, 5% CO2 incubator at 37°C. The keratinocyte monolayers were then washed thoroughly, and the cells were scraped with a rubber policeman; pelleted by centrifugation at 300 × g for 5 min at 4°C; resuspended in ice-cold 10 mM TBS containing 0.025% NaN3, 20 mM Ca2+; 1% Nonidet P-40 (Amershams Pharmacim Biotech) and the protease inhibitors 1 mM iodoacetamide, 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, and 5 μg/ml chymotatin; put on ice; and homogenized. Solubilized [35S]methionine-labeled proteins were separated by centrifugation at 40,000 × g for 5 min at 4°C and used as a source of naturally folded keratinocyte proteins. The radiolabeled keratinocyte protein solution was incubated with immunofinity-purified anti-PX PV IgG overnight at 4°C with gentle shaking. The immune complexes were precipitated with slurry protein A-Sepharose suspension, washed, and resolved by 7.5% SDS-PAGE. The gels were fixed and enhanced with 1 mM sodium salicylate, and the radioactivity was analyzed using the storage phosphor autoradiography feature of the Storm system (Molecular Dynamics, Mountain View, CA).

Antibody Transfer to Neonatal Mice—The PV phenotype was induced in neonatal mice by passive transfer of PV patients' serum IgG fractions to normal Balb/c mice (8). The IgGs were injected intraperitoneally through a 30-gauge needle at a dose of 20 mg/g of body weight per day into 10- to 12-h-old pups. The neonates always received the same amounts of PV IgG (experiment) and normal human IgG (control). The latter was isolated from normal human serum purchased from Sigma Chemical Co. The mice were sacrificed when fully developed skin lesions could be seen or, if no gross lesions could be observed, approximately 24 h after the last injection. The lesional and perilesional skin samples were collected and examined by staining with hematoxylin and eosin and by DIF with FITC-conjugated goat anti-human IgG antibody (Pierce).

Statistics—The results of quantitative experiments were expressed as mean ± S.D. Significance was determined using the Student's t test.

RESULTS

Selection of an Immunoaffinity-purified Acantholytic Anti-keratinocyte PV IgG as a Candidate for cDNA Library Screening—In an attempt to identify the pathogenic PV antibody, we investigated the ability of different fractions of immunoaffinity-purified anti-keratinocyte PV IgGs to: 1) stain the stratified epithelial substrates in a fishnet-like, “intercellular” pattern, which is diagnostic of PV (1); and 2) induce acantholysis in keratinocyte monolayers, which has become a standard approach to test disease-causing ability of PV antibodies (29, 30). Among tested PV IgG fractions, the antibody eluted from the horizontal strip excised from the 75-kDa area of the immunoblotting membrane produced intercellular epithelial staining of both normal human skin and monkey esophagus in IIF experiments (Fig. 1, A and B). Treatment of confluent monolayers of normal human KC with this immunoaffinity-purified PV IgG fraction, but not with normal human IgG, produced changes in keratinocytes, which is characteristic of pemphigus acantholysis (Fig. 1, C and D). No acantholysis could be seen in cultures treated with equal amounts of PV IgG eluted from the 130-kDa area of immunoblots of normal human keratinocyte proteins (data not shown). Therefore, PV IgG immunoaffinity-purified on a 75-kDa band was selected to probe the λgt11 human keratinocyte cDNA expression library.

Isolation of cDNA Clones Encoding PX and Sequence Analysis—Approximately 3 × 106 plaques of λgt11 human keratinocyte cDNA expression library were screened with the affinity-purified antibody from three PV sera (codes: PRC-45, PRC-46, and PRC-47), which contained the anti-75-kDa band acantholytic PV IgG that stained the stratified epithelium in a pemphigus-like pattern. In the first round of screening, four plaques were found to be positive for antibody binding. However, only two clones, designated as K5 and K12, remained immunoreactive after subsequent rescreening. Because PV IgG eluted from the filter blotted with both K5 and K12 clones stained monkey esophagus in a pemphigus-like pattern (data not show), both clones were selected for further characterization. PCR amplification of the cDNA insert using a pair of λgt11 cloning primer revealed that K5 and K12 clones carried the 1.3- and 1.4-kb cDNA inserts, respectively (Fig. 2 A). Unexpectedly, sequence analysis of the cDNA inserts from both clones predicted the same open reading frame of 1035 bp, encoding a full-length protein comprised of 345 amino acids (Fig. 2B) with a calculated molecular mass of 38.3 kDa. Examination of the nucleotide sequence revealed an in-frame stop codon situated upstream of the first ATG codon, which indicated that a complete coding region was identified. There were two tandem ATG potential translation initiation codons after the upstream in-frame stop codon. The first one most likely represented the initiation codon, because it was preceded with the Kozak consensus sequence (31). No poly(A) tail was detected. A BLAST search of the GenBank® data base at the NCBI web site showed that the nucleotide sequence encoded a previously unknown molecule. The deduced amino acid sequence revealed a high degree of homology to the members of the Ca2+-dependent annexin protein gene family. The strong-
Identification of pemphaxin (PX)—a novel human annexin-like molecule—using the anti-75-kDa band immunoaffinity-purified PV IgG as a probe.

A. PCR amplification of cDNA inserts from λgt11 phages isolated from the clones K5 and K12 using specific λgt11 forward and reverse cloning primers. The 1.5- and 1.6-kbp PCR products carried copies of 1.3- and 1.4-kbp cDNA inserts, respectively, from the two clones that were specifically recognized by affinity-purified PV IgG as a result of screening of 3 million plaques of a λgt11 human keratinocyte cDNA expression library. Sequence analysis of both cDNA inserts revealed that both encoded for the same novel molecule, PX.

B. The nucleotide sequence and the predicted amino acid sequence of PX. The in-frame upstream and downstream stop codons are underlined. The Kozak sequence that precedes the potential initiation ATG codon is double underlined. The Kozak sequence reported for different species showing that PX shares the same amino acids in most of the conserved regions. Shaded regions indicate the identical amino acid residues among all compared sequences. The arrow denotes potential glycosylation site. The asterisks denote the potential type II Ca\(^{2+}\) binding sites. The potential actin bundling site is underlined. Anx-2, annexin-2.
est similarity, approximately 40%, was observed with annexin-2 present in chicken (GenBank accession number P17785), cow (P04272), rat (Q07936) and humans (NP004030). The amino acid sequence alignment (Fig. 2C) revealed several conserved regions, including the type II Ca\textsuperscript{2+} binding sites (32, 33) and the actin bundling site that plays a role in Ca\textsuperscript{2+}-dependent bundling of actin microfilaments by annexins (34).

Because of its homology to the members of the annexin protein gene family, we tentatively named this newly discovered PV antigen pemphaxin (i.e. pemphigus annexin 5 pemphaxin).

Expression of the rPX-His Fusion Protein in E. coli and Its Affinity Purification—To allow experiments with immunoaffinity-purified anti-PX PV antibody, we produced a full-length recombinant PX. Because both K5 and K12 clones carried full-length cDNAs encoding the complete open reading frame of PX, we chose to directionally clone the K5 cDNA to the pQE-30 expression vector, which was designed to express PX protein carrying a poly-His tag at its N terminus. The cloned pQE-30-PX was transformed into E. coli M15 cells, and the colonies expressing rPX-His were selected by screening with anti-RGS-His monoclonal antibody. Antibody staining revealed six strongly positive colonies that contained correct PX inserts, as confirmed by subsequent sequencing. Clone 1 was selected for a time course characterization of PX expression. As seen in Fig. 3A, the transfected bacteria began to produce rPX-His after induction with 2 mM IPTG, and the amount of this fusion protein, estimated by the time course study with the time points of 1, 2, 3, and 4 h, gradually increased and reached saturation at 4 h after induction. As expected from the deduced molecular mass of PX, the newly produced rPX-His migrated with a 40-kDa protein band on the 12% SDS-PAGE gel. No proteins were induced by IPTG in control, non-transfected E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown). The rPX-His was isolated from the mixture of E. coli M15 cells (data not shown).

Cholinergic Radioligand Binding by rPX-His—Cholinergic ligand binding properties of annexins-1, -2, and -3 (35) suggested that PX also acts as a cholinergic receptor binding ACh on the cell surface of KC. To test this hypothesis, rPX-His was used in a standard radioligand binding assay. The saturable specific binding was achieved with the reversible cholinergic radioligand [\textsuperscript{3H}]ACh (Fig. 4A). The analysis of binding kinetics revealed the $K_d$ value of 909 nM and a $B_{\text{max}}$ of 176 pmol/mg of protein, indicating that, on the cell membrane of KC, PX may act as a low affinity receptor for endogenously produced and secreted ACh.

Because we demonstrated in a previous study (5) that 85% of pemphigus patients develop autoantibodies, which immuno-precipitate a keratinocyte membrane protein covalently labeled with the cholinergic radioligand [\textsuperscript{3H}]PrBCM, we further asked whether [\textsuperscript{3H}]PrBCM can specifically label rPX-His. The specificity of [\textsuperscript{3H}]PrBCM binding to rPX-His was demonstrated in the binding inhibition experiment using non-labeled cholinergic ligands ACh, nicotine, and muscarine as competitors (Fig. 4B). As expected, ACh as well as its nicotinic and muscarinic congeners decreased significantly ($p < 0.05$) the amount of [\textsuperscript{3H}]PrBCM bound to rPX-His, indicating that PX exhibits dual, muscarinic and nicotinic pharmacology. The dose-dependent radioligand binding inhibition assay with [\textsuperscript{3H}]PrBCM could not be performed because of the irreversible nature of its binding to a receptor molecule, via an alkylation reaction (36).

Characterization of Immunoaffinity-purified Anti-PX PV Antibody—The anti-PX PV IgG was immunoaffinity-purified on rPX-His immobilized on the Ni-NTA column via its His tags, and the PV IgG fraction eluted from the resin was characterized by: 1) IIF assay using human skin and monkey esophagus as substrates; and 2) immunoprecipitation assay with metabolically radiolabeled keratinocyte proteins. In the IIF assays, the immunoaffinity-purified anti-PX PV IgG stained, in a distinct fishnet-like, pemphigus pattern, the stratified squamous epithelium in human skin and monkey esophagus (Fig. 5, A and...
The epithelia of other types, such as those lining human bronchi, lung alveoli, small and large intestine, and renal glomeruli, did not exhibit specific staining (data not shown), indicating that the stratified epithelium is a major site of the epithelial expression of PX in human beings. We did not test non-epithelial tissues in this study.

Although addition of a 6xHis-tag to PX should not alter its conformational epitope, we sought to rule out even a remote possibility that, in addition to anti-PX, the rPX-His fusion protein absorbs antibodies of other specificities. The purity of PV IgG eluted from rPX-His was tested in a immunoprecipitation assay, which allows an antibody to recognize its antigen in the native form, to increase the sensitivity and specificity of antibody characterization. The immunoprecipitation assay showed that the affinity-purified anti-PX PV IgG precipitated keratinocyte proteins with apparent molecular masses of 40 and 80 kDa (Fig. 5C). Because the deduced molecular mass of PX is 38.3 kDa, these results suggested that PX exists as a monomer and a homodimer in KC. This hypothesis was further supported by demonstration of the predicted reciprocal changes in the relative amounts of the 40- and 80-kDa products depending on the presence or absence of the reducing agent β-mercaptoethanol in the SDS-PAGE buffer (Fig. 5C). Indeed, the covalent linkage of two annexins in a dimer is common for certain annexins (reviewed in Ref. 37).

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Absorption of Disease-causing PV Antibodies with rPX-His—To determine the pathophysiological significance of anti-PX antibody in pemphigus, we next asked if depletion of the PV IgG fraction of anti-PX antibody could affect the ability of PV IgG to cause gross skin blisters in neonatal mice. Equal amounts of the intact whole PV IgG fraction (positive control) and the PV IgGs that either passed through the Ni-NTA column containing immobilized rPX-His or were eluted from the column were injected intraperitoneally into 10- to 12-h-old Balb/c mice at a concentration of 20 mg of IgG/g of body weight per day. Only the mice that received non-absorbed PV IgGs reproducibly developed pemphigus-like gross skin lesions between the 16th and 24th h after a single injection. The mice injected repeatedly with either the pass-through (Fig. 5D) or the immunoaffinity-purified anti-PX IgGs (not shown) did not develop any macro- or microscopic skin changes, despite deposition of injected IgGs in mouse epidermis in both cases (Fig. 5D).
lytic activity of preabsorbed PV IgGs could be restored by adding back the adsorbed anti-PX antibody. As seen in Fig. 5 (F and G), the pups injected with the pass-through PV IgGs supplemented with anti-PX IgG eluted from the affinity column produced the PV phenotype that was indistinguishable from the epidermal acantholysis and gross skin blisters produced by non-adsorbed PV IgG (not shown). These results clearly indicated that, in addition to anti-PX antibody, the pool of disease-causing PV IgG contains autoantibodies to other keratinocyte self-antigens and suggested that a cumulative effect of anti-keratinocyte antibodies of different specificities is required to break up the integrity of live epidermis and induce skin blistering.

DISCUSSION

In this study we selected the PV IgG fraction that can both stain the epithelial substrates in the pemphigus-like pattern and induce acantholysis in keratinocyte monolayers to probe λgt11 keratinocyte cDNA library for novel targets of disease-causing PV antibodies. The PV antibody immunoaffinity-purified on a 75-kDa keratinocyte protein band identified a novel human annexin-like molecule, which we termed PX. Recombinant PX was produced and shown to bind specifically ACh and its nicotinic and muscarinic congeners. The obtained results indicate that PX may serve as a cell surface cholinergic receptor mediating a novel ACh signaling pathway involved in the physiological control of cell-to-cell adhesion and that autoimmunity to PX may lead to acantholysis.

Pemphigus is an autoimmune disease with a complex pathophysiology. Both humoral (38) and cellular (39) effectors of autoimmune aggression against KC are involved in the pathogenesis of this disease, and it has been demonstrated that local activation of trypsin-like serine proteases, such as plasminogen activator (40), complement (41), eicosanoids (42), and proinflammatory cytokines (29, 43), all can contribute to acantholysis. The precise mechanism leading to acantholysis in PV, however, is yet to be determined. It is currently held that an autoantibody to the 130-kDa adhesion molecule Dsg 3 causes pemphigus by disrupting directly the keratinocyte cell-to-cell bridges or desmosomes (44, 45). The intuitive notion that the disease of skin adhesion is caused by an antibody to the adhesion molecule, however, awaits its direct experimental confirmation. Meanwhile, Kitajima et al. (46) demonstrated that desmosome formation induced by switching the incubation medium from a low to a high Ca2+ content is not inhibited by the binding of PV IgG to the cell membrane of cultured KC. In agreement with this report, we could not detect any morphological changes in the keratinocyte monolayers treated with the anti-130-kDa PV IgG for 16 h, whereas the acantholysis in cell monolayers usually develops within 12 h after addition of the whole PV IgG fraction (20, 29, 30). Fan et al. (47) attempted to create an animal model of PV by immunizing four different strains of mice, Balb/c, DBA/1, SJL/J, and HRS/J, with full-length Dsg 3 protein, recombinant extracellular portion of Dsg 3, and the synthetic peptides spanning the entire Dsg 3. However, they found no signs of pemphigus, oral or cutaneous, in any of the animals, despite relatively high, up to 1/2560, titer of anti-Dsg 3 antibody. Even after the immune sera were concentrated 10-fold and inoculated into neonatal mice, the mice of only one strain, Balb/c, developed the lesion. These results demonstrated that, at the serum titers that are equivalent or exceeding those found in PV patients, the anti-Dsg 3 antibody is not sufficient to cause pemphigus symptoms. The suprapharmacological doses of this antibody, however, can physically interfere with cell-to-cell adhesion, as illustrated by the occurrence of microscopic changes in the oral mucosa of immuno-
deficient Rag-2 knockout mice grafted with a spleen producing anti-Dsg 3 antibodies (48). Unfortunately, the interpretation of findings in mice with adoptively transferred anti-Dsg 3 antibodies in the study of Amagai et al. (48) is complicated by its rather controversial nature, which includes direct conflict with the existing data. For instance, according to Amagai et al. (48), lack of skin changes in Balb/c mice immunized with Dsg 3 is attributed to inability of this strain of mice to produce anti-Dsg 3 antibody titers in these animals, albeit without any mucocutaneous signs of PV. Furthermore, Amagai et al. (48) opine that, by analogy with the interpretation of the Dsg3null phenotype (18), a transient hair loss accompanied by transient microscopic alterations of keratinocyte adhesion in the oral cavity, which is all that can be observed in the recipient Rag-2–/– mice, should be interpreted as the PV phenotype. However, the following facts argue against this interpretation: 1) hair loss is not a sign of PV (1); 2) true PV is a disease severe enough to kill approximately 90% of patients, if left untreated (reviewed in Ref. 2); and 3) neither recipient Rag-2–/– mice nor Dsg3null mice develop spontaneous skin blisters (5, 15, 18). Nevertheless, the notion about the pathophysiologic significance of Dsg 3 antibody in PV has been supported by the results of in vivo experiments in which pemphigus antibodies affinity-purified on the rDsg3-Ig chimera induced gross skin blisters in neonatal mice (13, 14). Unfortunately, the profile of PV IgGs adsorbed by the rDsg3-Ig-His baculoprotein has never been shown, leaving unresolved the purity and specificity of the antibodies used in the passive transfer experiments. Therefore, we had to characterize the antigenic reactivity of PV IgG absorbed with rDsg3-Ig-His in our laboratory (19). We established that the antibodies adsorbed on rDsg3-Ig-His are directed toward several keratinocyte proteins, including an unknown 130-kDa self-antigen recognized in the Western blot of keratinocyte proteins of Dsg3null mice.

To select the PV IgG fraction that most likely contains disease-causing antibody, we screened PV IgG fractions eluted from different areas of the immunoblotting membrane for their ability to both: 1) stain epidermis in a fishnet-like, pemphigus pattern; and 2) produce acantholysis in keratinocyte monolayers. The anti-75-kDa band PV IgG met both criteria. Failure of the antibody eluted from the 130-kDa area of the immunoblotting membrane to fulfill both criteria was not surprising, because in the past this antibody was selected for the cDNA screening experiments that identified Dsg 3 based on the first criteria only (9). In our study, anti-75-kDa band PV IgG antibody caused acantholysis, which could be observed at 0.09 mM Ca²⁺ in KGM. Although expression of Dsg 3 in KC requires preincubation of the cells at high, from 1.8 to 2.55 mM, extracellular Ca²⁺ (9, 49, 50), we and other workers have previously demonstrated that binding of disease-causing PV IgGs to KC and acantholysis in cell monolayers both occur at as low as 0.1 mM Ca²⁺ (20, 29). This fact suggests that, in addition to blocking the “adhesive sites” of desmosomal cadherins with anti-Dsg PV IgG, binding of pemphigus antibodies to KC initiates an intracellular signaling cascade that can lead to disassembly of other types of intercellular junctions comprised of classical cadherins, such as tight junctions, adherence junctions, and gap junctions, all of which can mediate keratinocyte cell-to-cell adhesion at low Ca²⁺ (51–53).

Screening of the Δg11 keratinocyte cDNA expression library with the acantholytic anti-75-kDa band PV antibody identified PX, a novel human annexin-like molecule. It appeared that two of 3 × 10⁶ plaques labeled with PV IgGs carried cDNA encoding for the same previously unknown annexin-like molecule with the predicted molecular mass of the translated product of 38.3 kDa. Sequence alignment with known annexins showed that PX shares the same amino acids in most of the conserved regions and is ∼40% similar to annexin-2. Annexin-2 may exist as a monomer, dimer, heterodimer, or heterotetramer in which two annexin-2 molecules combine with two smaller subunits, p11, that resemble the S-100 protein of the calmodulin family (54). Because the PV IgG immunoaffinity-purified on rPX-His labeled keratinocyte proteins with apparent molecular masses of 40 and 80 kDa, it can be postulated that PX forms homodimers.

Annexins comprise a unique family of Ca²⁺- and phospholipid-binding proteins encoded by some 20 different genes, which are ubiquitous among eukaryotic organisms, single-celled organisms, and plants and animals (reviewed in Refs. 55, 56). Individual annexins have been described under the names anchoring, calcineurin, calelecin, calpactin, calphobindin, chromobindin, endonexin, lipocortin, and synexin. Different annexins have been shown to: 1) participate in ligand-mediated cell signaling both directly, by forming Ca²⁺-sensitive, voltage-gated Ca²⁺ channels, and indirectly, by generating membrane-derived second messengers; 2) mediate anti-inflammatory action of glucocorticosteroids via inhibition of phospholipase A₂; 3) regulate and directly mediate cell-to-cell adhesion; 4) mediate endo- and exocytosis; 5) inhibit blood coagulation; 6) regulate Ca²⁺-dependent Cl⁻ conductance; and 7) participate in the processes of cell proliferation, apoptosis, and virus infection (reviewed in Refs. 37, 57–60). PX turned out to be a sixth protein of the annexin protein gene family identified in normal human skin to date. Annexins-1, -2, -5, -6, and -7 have been demonstrated previously (61, 62). Expression of annexins in epidermis is differentiation-dependent (63). Annexin-1 immunoreactivity is found almost entirely around the perimeter of KC, especially tonofilament/desmosome-rich prickle KC (61). It has been noted that raising intracellular Ca²⁺ results in peripheral relocations of annexins-2, -4, -5, and -6 from the perinuclear areas (64). Annexin-2 has been shown to be directly involved in regulation of cell adhesion and migration (65). The presence in PX of the conserved sites providing for Ca²⁺ binding and for bundling of actin filaments suggests that PX, just like annexin-2, regulates assembly and maintenance of the cytoskeletal units. This actin polymerization is now believed to play a crucial role in epithelial cell-to-cell adhesion, because disruption of this process in an animal model causes skin lesions indistinguishable from PV lesions (66).

Although annexins lack a leader sequence (and do not pass the Golgi apparatus), they are found on the keratinocyte cell surface, where they can function as receptors. Extracellular annexins have been demonstrated to bind collagen, tenasin, and plasminogen activator (65, 67–70). Binding of tenasin-C to annexin-2 provokes three cellular responses: loss of adhesion, lateral migration, and enhanced cell division (71). Tenasin expression is induced in pemphigus skin as well as in the skin of other blistering dermatoses (72).

To characterize PX, we produced full-length recombinant protein using pQE-30 vector, which contained IPTG-inducible promoter transformed into the E. coli M-15 competent cells. Plasmid purified from this clone was analyzed by restriction enzyme analysis and sequencing. Both confirmed that the PX DNA insert was 100% correct. The rPX-His was affinity-purified and used in standard receptor-ligand binding assays with the cholinergic radioligand [³H]ACh. The analysis of the saturable binding of [³H]ACh showed that PX can function as a low affinity cholinergic receptor on the cell membrane of KC. These results were expected, because choline, which itself serves as a pharmacological agonist of cholinergic receptors (73, 74), has
been shown to specifically bind to annexin-1, -2, and -3 (35). Likewise, rPX-His could be specifically tagged with a covalent cholinergic radioligand [³²P]PrBCM, which was previously used by us to label keratinocyte membrane proteins immunoprecipitated by 85% of pemphigus patients (5).

The results of pharmacological experiments demonstrated that rPX-His exhibited conformational structure, thus allowing specific binding of cholinergic ligands. Post-translational modification is not required for ligand binding to single-unit ACh receptors, such as the muscarinic receptor (75, 76). However, the affinity of ACh binding by the wild-type PX, which can forms dimers, may be different from that shown by rPX-His in vitro, because the bacterial system in which it was expressed was not capable of post-translational modification, such as glycosylation, which is known to play an important role in ligand binding by multi-subunit ACh receptors such as the nicotinic receptor (77). Thus, PX can act as a novel keratinocyte cell surface receptor for the cyto transmitter ACh, synthesized and secreted by human KC in autocrine and paracrine fashions, and mediate known effects of ACh and cholinergic drugs on keratinocyte adhesion (reviewed in Refs. 21, 22). PX can also represent, at least in part, the putative keratinocyte cholinergic receptors targeted by PV IgG (5, 20).

The drugs that act at keratinocyte cholinergic receptors have been shown to alter cell motility and adhesion. Exposure of suspended KC to ACh results in attachment and spreading of the cells on the dish surface and development of intercellular contacts within 20–30 min, whereas non-stimulated cells accomplish this process within 90–120 min. On the other hand, exposure of a confluent keratinocyte monolayer to pharmacological antagonists of ACh leads to a characteristic acantholytic response. The cells retract their cytoplasmic projections, lose cell-to-cell attachments, detach from each other, and become round in shape and non-motile—characteristics that remarkably resemble pemphigus acantholysis in vitro (20). We have previously reported that ACh and its muscarinic and nicotinic congeners can prevent and reverse acantholysis produced in keratinocyte cultures by PV IgG (20). A receptor/ligand type of interaction of disease-causing PV IgG, with its target being a keratinocyte cell membrane protein, was first proposed by Patel et al. (78) based on the results of time-course study of the fate of the PV antibody/antigen complex. A direct evidence of activation of second messenger systems in response to PV IgG binding to KC have been obtained in the studies showing changes with phospholipase C, inositol 1,4,5-trisphosphate, transmembrane flux and intracellular levels of Ca²⁺, intracellular cAMP/cGMP ratios, and activity and intracellular location of protein kinase C (reviewed in Refs. 5, 79). Therefore, binding of anti-PX antibody to KC may lead to acantholysis by competing with the natural agonist ACh, thus interrupting physiological regulation of keratinocyte adhesion. In keeping with the notion that autoantibody-mediated ligation of PX on the cell membrane of KC can alter the cell adhesive function are the results showing that an antibody to annexin-2 inhibits cell-to-cell attachment (80).

To determine the role of anti-PX antibody in pemphigus pathophysiology, we preabsorbed PV sera with rPX-His and tested acantholytic activities of both the PV IgGs depleted of anti-PX antibody and the PV IgG eluted from rPX-His. Neither IgG fraction could induce micro- or macroscopic mucocutaneous lesions in neonatal Balb/c mice. Addition of the adsorbed anti-PX PV IgG to the preabsorbed IgG fraction restored its acantholytic activity. These findings suggested that anti-PX antibody is one of the major contributors to skin blistering in PV patients. The fact that anti-PX PV antibody alone was sufficient to cause acantholysis in vitro (Fig. 1) but could not do so in vivo was not surprising. Obviously, the cell-to-cell adhesion of KC cultured at low Ca²⁺ is less sophisticated than that taking place in live epidermis, with regard to a variety of adhesion molecules and control mechanisms, which include local anti-acantholytic factors such as interleukin-10 (30). Needless to say, the integrity of the epidermal barrier in higher species relies on more than a single molecule. For example, inactivation of an adhesion molecule such as Dsg 3 does not lead to skin blisters and is well compatible with the normal life span of Dsg3null mice (5, 18), whereas a loss of immunological tolerance to keratinocyte self-antigens in PV is potentially lethal in 90% of patients (reviewed in Ref. 2). Therefore, to explain clinical and immunological correlations in PV, we propose a “multi-hit” hypothesis, which postulates that acantholysis in PV results from simultaneous and cumulative effects of autoantibodies directed toward different keratinocyte self-antigens, including the “structural” antigens, such as desmosomal cadherins, and “functional” antigens, such as cell surface receptors regulating function of the adhesion and cytoskeletal units.

The rationale behind our emphasis on the importance of “functional” targets of PV autoimmunity stems from recent discoveries of the genetic defects that underlie certain skin diseases. For instance, patients with genetic defects of the adhesion molecules Dsg 1 and desmoplakin develop neither macroscopic nor light- or electron-microscopic alterations of keratinocyte cell-to-cell adhesion but produce instead a pal moplantar keratoderma, represented by linear and focal hyperkeratosis on palms and soles (81–83). In marked contrast, intra-epidermal split and PV-like skin lesions in patients with keratosis follicularis, or Darier-White disease, and patients with benign familial pemphigus, or Hailey-Hailey disease, result from a mutation in the genes coding for Ca²⁺ pumps, the ATP2A2 and ATP2C1, respectively (84, 85). Calcium metabolism in the epidermis of PV patients may also be altered. We have recently found that PV patients develop autoantibodies to the novel human α9 ACh receptor subunit that comprises ACh-gated Ca²⁺ channels on the cell membrane of human KC (19).

In summary, in this study we identified PX, a novel annexin-like molecule, which can function as a keratinocyte cholinergic receptor mediating biological effects of ACh on KC, including regulation of cell-to-cell adhesion. PX is targeted by PV autoimmunity and may represent one of the major targets for acantholytic autoantibodies. Further studies should be directed to elucidate the biochemical mechanisms by which the anti-PX antibody alters keratinocyte adhesion in vitro and the biological effect(s) caused by cholinergic ligand binding to PX. Furthermore, because annexins are well known mediators of anti-inflammatory effects of glucocorticosteroids in the skin (86), and because glucocorticosteroids can directly protect KC from the acantholytic effect of PV IgG in vitro (87), it will be important to elucidate possible relationships between the effects of glucocorticosteroids on PX and keratinocyte adhesion. Such an association may lead toward development of non-hormonal treatment of PV, because cholinergic drugs that, just like glucocorticosteroids, exhibit direct anti-acantholytic activity (20) may do so by competing with PV IgG for binding to PX on the cell membrane of KC.

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