

HUMAN AUTOANTIBODIES AGAINST A DESMOSOMAL  
PROTEIN COMPLEX WITH A CALCIUM-SENSITIVE  
EPI TOPE ARE CHARACTERISTIC OF PEMPHIGUS  
FOLIACEUS PATIENTS

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Pemphigus is a group of autoimmune skin diseases in which autoantibodies develop against the cell surface of keratinocytes and intraepidermal blisters occur as a result of the loss of epidermal cell cohesion. There are two distinct forms of pemphigus, pemphigus foliaceus (PF) and pemphigus vulgaris (PV). Autoantibodies from PF patients do not bind the specific keratinocyte cell-surface glycoproteins defined by PV autoantibodies (1). Immunohistochemical morphologic studies (2) of mouse keratinocytes in culture have suggested that certain pemphigus patients may have antibodies against desmosomes. Immunoblotting studies (3) have indicated that a subgroup (about one-third) of PF patients, but not PV patients, have antibodies against denatured desmoglein I (DGI), a core desmosomal glycoprotein of  $M_r$  160,000. However, the majority of PF patients' autoantibodies did not bind any specific polypeptides in immunoblotting experiments, which suggests that either these antidesmosomal antibodies are not characteristic of all PF patients or that, if they are, the majority of patients' antibodies no longer bind to desmosomal proteins once they are denatured for immunoblotting studies. Thus, to determine if antibodies to desmosomal polypeptides are present in all PF patients, we used a minimally denatured nonionic detergent extract of human epidermis in an immunoprecipitation procedure with PF antibodies.

**Materials and Methods**

*Antisera.* Serum was obtained from patients with PF (4), bullous pemphigoid (an autoantibody-mediated subepidermal blistering disease), Darier's disease (a nonimmunologic hereditary disease in which there is loss of epidermal cell cohesion), and from normal human volunteers. A monospecific rabbit polyclonal antiserum (called R<sub>p</sub>DGI-1) raised against denatured DGI has been previously characterized (3, 5).

*Extraction of Epidermis.* As previously described (6), four suction blisters were raised on the anterior forearms of normal human volunteers. The roofs of the blisters, which consisted of epidermis only (area ~3 cm<sup>2</sup>), were extracted by sonication and vortexing in 2 ml of 0.5% NP-40 in Tris-buffered saline (TBS) with 1 mM PMSF (as a protease inhibitor) and, unless stated otherwise, 2 mM CaCl<sub>2</sub>. Membrane fragments, subcellular

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particles, and insoluble proteins were removed by centrifugation for 1 h at 100,000 *g*. To remove protein A-binding Igs, the supernatant was then absorbed sequentially with two pellets each prepared from 1 ml of a 10% suspension of protein A-coated staphylococci (Pansorbin from Calbiochem-Behring Corp., La Jolla, CA). The solubilized proteins were then radiolabeled with  $^{125}\text{I}$  using Iodo-beads (Pierce Chemical Co., Rockford, IL). Unbound  $^{125}\text{I}$  was removed by dialysis against 0.3% NP-40 in TBS, with 2 mM  $\text{CaCl}_2$  added if  $\text{CaCl}_2$  was in the extracting solution.

**Immunoprecipitation.** Antigen-antibody complexes were precipitated with protein A-bearing staphylococci. The immunoprecipitation procedure was as previously described (1) except as follows. Each immunoprecipitation used  $15\text{--}20 \times 10^6$  cpm of radiolabeled epidermal protein and 10  $\mu\text{l}$  of whole serum. If  $\text{CaCl}_2$  was used in the epidermal extracting solution then 2 mM  $\text{CaCl}_2$  was added to all buffers. Immunoprecipitated polypeptides were then solubilized in sample buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 0.1 M DTT) and separated by SDS-PAGE or, in two dimensions, by isoelectric focusing followed by SDS-PAGE (3). Radiolabeled polypeptides were visualized by autoradiography performed with intensifying screens.

### Results and Discussion

When extractions and immunoprecipitations were performed with 2 mM  $\text{Ca}^{2+}$  added to all buffers, all sera from 15 different PF patients immunoprecipitated, in a stoichiometric ratio, a complex of polypeptides that, when separated by SDS-PAGE, dissociated into three major polypeptides of  $M_r$  260,000, 160,000, and 88,000, and a minor polypeptide of  $M_r$  110,000 (Fig. 1 A). Controls that included sera from 18 normal humans, 7 patients with bullous pemphigoid, and 2 patients with Darier's disease failed to immunoprecipitate any specific proteins. These findings demonstrate that all PF sera tested bind a characteristic set of polypeptides extracted from normal human epidermis.

If calcium was not added to the extractant or if the added calcium was removed (after extraction of the epidermis) by chelation with EDTA, then only three of seven PF sera tested reacted with the protein complex. The three sera that reacted in the absence of calcium were shown previously (3, 4) to bind to denatured DGI by immunoblotting, whereas the four sera that failed to precipitate the complex without added calcium did not bind to denatured DGI by immunoblotting (PF sera that have been shown previously to bind DGI by immunoblotting will be referred to as blot<sup>+</sup>, and those shown previously to be negative by immunoblotting will be called blot<sup>-</sup>). Fig. 2 shows two such representative sera, PF 572 is blot<sup>-</sup> and PF 573 is blot<sup>+</sup>. PF 572 no longer precipitates the complex in the absence of calcium. The complex is still present and not merely degraded by proteases in the absence of added calcium because PF 573 (blot<sup>+</sup>) still immunoprecipitates the complex when calcium is removed. These findings show that blot<sup>-</sup> PF antibodies bind a calcium-sensitive epitope on the complex of polypeptides, but that blot<sup>+</sup> sera, which are capable of binding denatured DGI (the 160-kD band of this complex; see below), can still bind this complex in the absence of calcium. These results suggest that blot<sup>-</sup> PF sera (which are the majority) bind to a conformational epitope, which is lost in the absence of calcium.

Because at least some PF sera have been previously shown to bind to DGI (3), we wanted to determine whether the protein complex precipitated by PF sera was related to desmosomes. Specifically, we suspected that the immunoprecipitated 160-kD polypeptide was DGI. To investigate this possibility, we used a

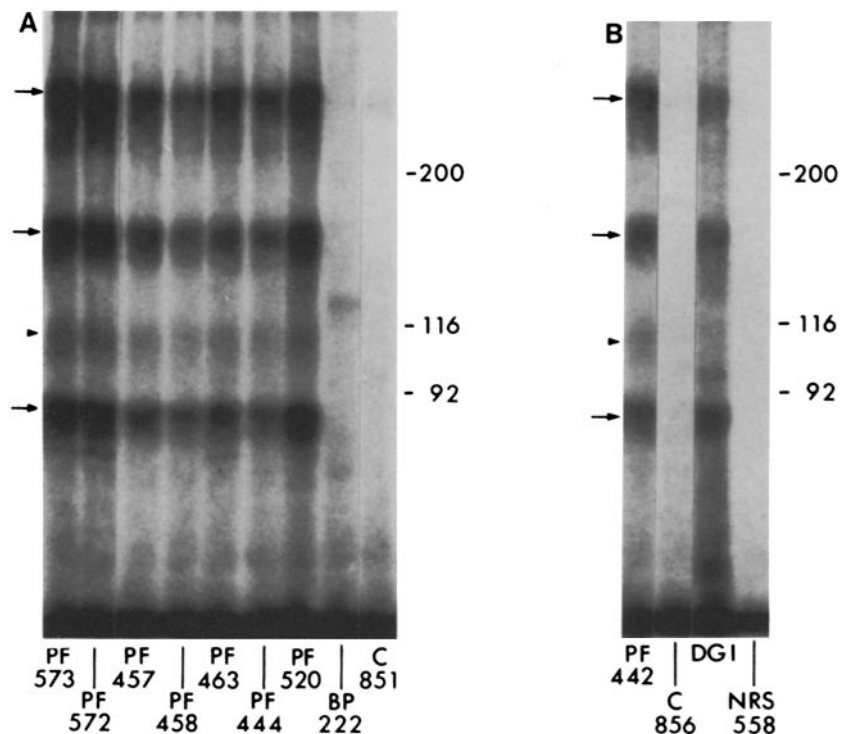


FIGURE 1. PF sera and antiserum against DGI immunoprecipitate a characteristic complex of polypeptides from calcium-containing nonionic detergent extracts of normal human epidermis. Each lane represents an immunoprecipitate of  $^{125}\text{I}$ -labeled human epidermal proteins, analyzed by SDS-PAGE and autoradiography and performed with either PF serum, a rabbit antiserum against denatured DGI, or as controls, bullous pemphigoid (BP) serum, sera from patients with Darier's disease (C), or normal rabbit serum (NRS). (A) All seven PF sera shown here, but none of the control sera, specifically immunoprecipitate four polypeptide bands, in a stoichiometric ratio. (B) Antiserum to DGI (lane marked DGI) precipitates the same complex as PF sera. Long arrows point to the three major polypeptides with  $M_r$  of 260,000, 160,000, and 88,000. The arrowhead points to a minor polypeptide of  $M_r$  110,000. The  $M_r \times 10^{-3}$  of concurrently run standards of known mass are shown on the right.

rabbit antiserum raised against denatured DGI in this immunoprecipitation assay. In the presence of calcium, PF autoantibodies and this rabbit anti-DGI immunoprecipitated the same polypeptide complex (Fig. 1 B). The rabbit antisera to DGI also precipitated the complex in the absence of calcium (data not shown), presumably because, like PF blot<sup>+</sup> sera, it is capable of binding denatured DGI, so any changes in conformation induced by the lack of calcium would not affect its antibody binding.

To further demonstrate that the rabbit anti-DGI and PF sera were precipitating the same complex, the immunoprecipitated proteins were separated in two dimensions by isoelectric focusing followed by SDS-PAGE. The major polypeptides precipitated by both the PF patients' sera and the anti-DGI serum comigrate (Fig. 3). The precipitated DGI has approximately the same molecular weight and isoelectric point as reported for bovine snout DGI (also called band 3). Although the other bands in the complex have not yet been identified, we speculate that they might be other desmosomal proteins, based on their molecular weights and

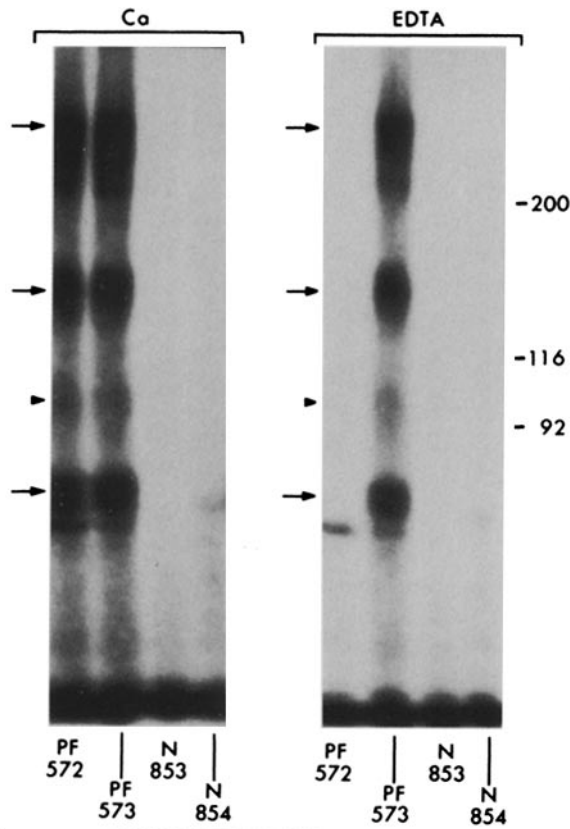


FIGURE 2. Immunoprecipitation of the polypeptide complex by certain PF sera is calcium dependent. Normal epidermis was extracted with added 2 mM  $\text{CaCl}_2$ . An aliquot of the extract was treated with 4 mM EDTA and immunoprecipitation was performed on both samples with PF sera or with normal human sera (*N*) controls. Both PF sera shown here precipitated the characteristic complex of polypeptides (260,000, 160,000, 88,000  $M_r$ , at arrows; 110,000  $M_r$ , at arrowhead) from the extract with added  $\text{CaCl}_2$  (*Ca*). In contrast, PF serum 572 no longer precipitates the complex from the EDTA-treated extract (*EDTA*). However, PF serum 573, which is known to react with denatured DGI (4), a 160,000  $M_r$  core desmosomal glycoprotein (identical to the 160,000  $M_r$  band shown here; see Fig. 1B), still was able to precipitate the complex in the absence of calcium. When epidermis was extracted without added EDTA and without added  $\text{CaCl}_2$ , results identical to those shown here with EDTA added to the  $\text{CaCl}_2$ -containing extract were obtained.  $\text{CaCl}_2$  alone or  $\text{CaCl}_2$  in excess of EDTA in the epidermal extracting solution permitted all PF sera tested to bind the polypeptide complex.

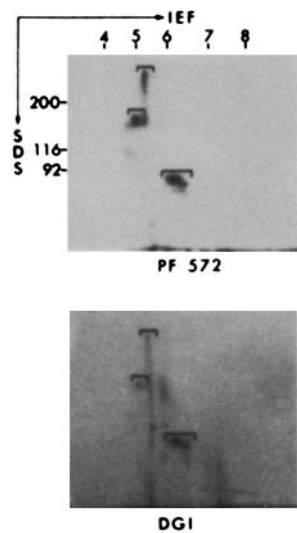


FIGURE 3. Two-dimensional gel electrophoresis, isoelectric focusing (*IEF*) followed by SDS-PAGE, of the polypeptide complex immunoprecipitated by PF serum and rabbit anti-DGI. Immunoprecipitated  $^{125}\text{I}$ -labeled proteins extracted (with added calcium) from normal human epidermis were separated by two-dimensional gel electrophoresis. Autoradiograms of these gels are shown. The numbers at the top of the autoradiogram represent the pH of the *IEF* gel run in the horizontal direction. The numbers on the side of the gel correspond to the  $M_r \times 10^{-3}$  of standards of known mass separated in the vertical direction by SDS-PAGE. The major polypeptides (*brackets*) immunoprecipitated by both sera PF 572 and the rabbit anti-DGI antiserum (*DGI*) have the same charge and molecular weight.

isoelectric points compared with those of the following bovine snout desmosomal proteins: desmocalmin:  $M_r$  240  $\times 10^3$ , pI 5.5; desmoglein II (also called band 4 or desmocollin):  $M_r$  100–115  $\times 10^3$ , pI 4.9; and desmoplakin III (also called

band 5):  $M_r$  83–86  $\times 10^3$ , pI 6.3–6.4 (5, 7–9).

Although the 240,000, 110,000, and 88,000  $M_r$  polypeptides are complexed tightly to DGI in nonionic detergent solution, these polypeptides are not all linked covalently because when exposed to 1% SDS they dissociate, and the PF blot<sup>+</sup> sera immunoprecipitate only DGI (data not shown).

The calcium sensitivity of an epitope for PF sera in the desmosomal complex is interesting in light of the importance of calcium in desmosomal formation (10, 11). Calcium, even in the absence of protein synthesis, can induce desmosomal formation in cultured epidermal cells (10). Because this calcium sensitive epitope defined by PF sera might be important in desmosomal assembly, we would speculate that the antidesmosomal antibodies, found in PF patients' sera, may contribute to the loss of epidermal cell cohesion by interfering with desmosomal formation.

### Summary

Pemphigus foliaceus (PF) patients have antibodies against a tightly, but non-covalently bound complex of polypeptides, which consists of desmoglein I (DGI) and other, possibly desmosomal, proteins. Most PF antibodies bind a calcium-sensitive epitope on this complex and chelation of calcium destroys the reactivity of these sera with the complex, but not the complex itself. The PF sera that do bind the desmosomal complex in the absence of calcium are those sera capable of binding denatured DGI on immunoblotting, and these same sera also immunoprecipitate only DGI when the desmosomal complex is dissociated with SDS. These findings demonstrate that autoantibodies against a complex of desmosome-associated proteins are characteristic of PF and define a calcium-sensitive conformational epitope on this complex.

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