BJD
British Journal of Dermatology

Immune response towards the amino-terminus of desmoglein 1 prevails across different activity stages in nonendemic pemphigus foliaceus

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Accepted for publication
4 February 2010

Key words
desmoglein 1, epitopes, pemphigus foliaceus

Conflicts of interest
None declared.

DOI 10.1111/j.1365-2133.2010.09696.x

Summary

Background Pemphigus foliaceus (PF) is a blistering skin disease mediated by antibodies to desmoglein (Dsg) 1. The two major subtypes are nonendemic and endemic PF. A previous study in endemic PF demonstrated that changes in antibody epitope could modulate disease relapse and remission.

Objectives To characterize the frequency of immunoreactivity to various Dsg1 extracellular (EC) domains in nonendemic PF and to study if there is any change in epitope profile across various activity stages.

Methods Sera from 34 patients with nonendemic PF were selected. To map the conformational epitopes by immunoprecipitation-immunoblotting, we constructed five Dsg1/Dsg2 domain-swapped molecules, with each molecule representing one EC domain of Dsg1 on a backbone of Dsg2.

Results Dsg1 EC1, EC2, EC3, EC4 and EC5 domains were recognized by 88%, 50%, 13%, 22% and 0% of active PF sera, respectively. Immunoreactivity to EC3 or EC4 often cosegregated with that to either EC1 or EC2. Longitudinal follow-up of 21 patients with PF for a median of 16 months revealed that, in most cases, immunoreactivity to the amino-terminus of Dsg1 persisted across various activity stages; only two patients lost their EC1 reactivity upon remission and changed their major epitope(s) to EC2 ± EC3.

Conclusions Most of the anti-Dsg1 antibodies in nonendemic PF bind to the amino-terminus of Dsg1, a region critical for intercellular adhesion of cadherins, and this skewed amino-terminal immunoreactivity prevails across various activity stages in most patients, even upon remission. These findings are valuable for understanding the biology of Dsg-mediated cellular adhesion as well as for the development of epitope-based monitoring and therapeutic strategies.

Pemphigus foliaceus (PF) is an autoimmune blistering skin disease characterized clinically by superficial cutaneous erosions, histologically by subcorneal acantholysis and immunologically by the development of autoantibodies against desmoglein (Dsg) 1.[1] The most common subtype is nonendemic PF, whereas endemic PF is found in rural areas of Brazil, Tunisia and Colombia.[2] Dsg, a transmembrane desmosomal glycoprotein, belongs to the cadherin superfamily of adhesion molecules. Up to now, four isoforms of Dsg have been described in humans. Ample evidence suggests that anti-Dsg1 antibodies are directly involved in the pathogenesis of PF.[1] Dsg4 immunoreactivity, although present in PF, represents only cross-reactivity with a subset of anti-Dsg1 autoantibodies.[3] In contrast, Dsg2 reactivity is not found in PF or pemphigus vulgaris (PV).[4]

Immunoreactivity of pemphigus sera to Dsg is conformational-sensitive as previous studies have shown reduced immunoadsorption of pemphigus sera by truncated extracellular (EC) domains of Dsg or if recombinant Dsg is denatured by low pH or heat.[5–7] These conformational epitopes are calcium dependent as ethylenediaminetetraacetic acid can abolish the ability of recombinant Dsg to block immunofluorescence staining by pemphigus sera.[8] Thus, previous attempts in

conformational epitope mapping in pemphigus have employed Dsg1/Dsg3 domain-swapped molecules.

Similar to other cadherins, the EC portion of Dsg is divided into five domains, EC1 (amino-terminus) to EC5 (carboxy-terminus). Previous studies, involving either immunoadsorption or generation of monoclonal antibodies (mAbs) and monoclonal single-chain variable fragments (scFvs), have shown that the amino-terminus of Dsg contains the most critical epitopes for pathogenic antibodies in pemphigus.[9–13] An early report showed that in endemic PF, Dsg1 autoimmunity evolved from nonpathogenic, towards EC5, to pathogenic, towards EC1–2; furthermore, immunoreactivity shifted back to EC5 upon remission.[10] In this study, we constructed a set of Dsg1/Dsg2 domain-swapped molecules, representing various EC domains of Dsg1 on a backbone of Dsg2. We aimed at characterizing the frequency of immunoreactivity towards various Dsg1 EC domains and investigating whether the epitope profile would change according to disease activity in nonendemic PF. Our results show that Dsg1 EC1 and EC2 harbour the major epitopes of sera taken from patients with nonendemic PF in both active stage and remission, and that amino-terminal Dsg1 reactivity is prevalent across various activity stages, even upon remission, in most patients with nonendemic PF. These findings are valuable for understanding the biology of Dsg-mediated cellular adhesion as well as for the development of epitope-based monitoring and therapeutic strategies.

Materials and methods

Human pemphigus foliaceus sera and disease activity scale

Sera were obtained from patients with nonendemic PF diagnosed clinically and histopathologically. They all had positive anti-Dsg1 antibodies but were negative for anti-Dsg3 antibodies by enzyme-linked immunosorbent assay (ELISA). To facilitate detection of epitopes by immunoprecipitation-immunoblotting (IP-IB), 34 patients with PF having Dsg1 ELISA indexes above 40 were selected. Among these, we chose 21 patients whose Dsg1 ELISA indexes were above 40 across various activity stages and performed a follow-up study on their epitope profile. In total, 91 sera were examined from these 34 patients with PF. All serum samples were stored at −30 °C until assayed.

Disease activity was defined by an arbitrary scale: remission, no active clinical lesion observed for at least 1 month; mild, 10% or less of skin surface involved and no functional disability; moderate, up to 30% of skin surface involved and moderate functional impairment; severe, greater than 30% skin surface involved and severe functional disability.[14,15] Active disease was defined as any stage ranging from mild to severe.

Enzyme-linked immunosorbent assay

Dsg ELISA was performed as previously described for all PF sera.[16] For sera showing ELISA indexes above 120, further dilution was made to determine the true index.[15]

Indirect immunofluorescence test

We determined the indirect immunofluorescence (IIF) titres of at least two sera representing different activity stages for each patient with PF whose epitope profile was monitored longitudinally, using cryosectioned normal human skin as substrate. PF sera were diluted serially from 1 : 10 up to 1 : 10 240 in phosphate-buffered saline with 1 mmol L^{-1} $CaCl_2$. Antibodies bound on the substrate were detected by using fluorescein isothiocyanate-conjugated antihuman IgG (Dako, Glostrup, Denmark) diluted at 1 : 100.

Plasmid constructs

We previously constructed recombinant molecules containing EC1–5 of Dsg1 and Dsg2 with E-tag and His-tag engineered at the carboxy-terminus, namely Dsg1-His and Dsg2-His.[4,14] We further constructed five domain-swapped molecules representing EC1–5 of Dsg1 on a backbone of Dsg2 (Fig. 1).

To build the domain-swapped molecules, respective primers (Table 1) were employed in polymerase chain reaction (PCR) reactions using either pEVmod-PFIg (GenBank X56654) or pQE-hDsg2 (GenBank Z26317) as templates.[4,7] The primers were designed to include nucleotides representing a cutting

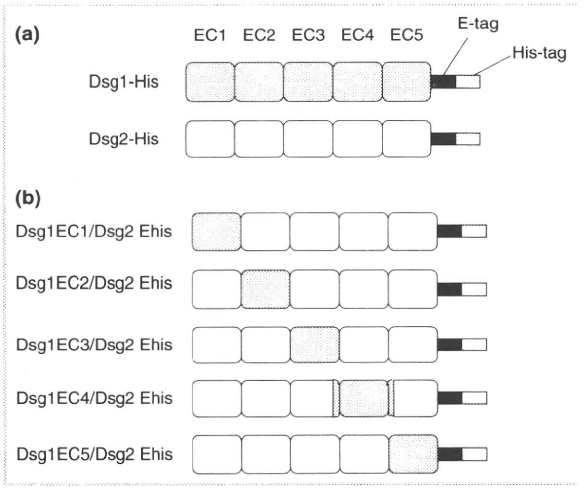

Fig 1. Schematic representation of molecular construct of recombinant molecules. (a) The whole extracellular (EC) portions of human desmoglein (Dsg) 1 and Dsg2, which were further subdivided into five domains, were used as positive and negative controls in immunoprecipitation-immunoblotting experiments. (b) EC1–5 domains of human Dsg1 were domain-swapped with the corresponding domains of human Dsg2 to generate five recombinant molecules. All recombinant molecules swapped exactly at the interface adjoining successive EC domains except for Dsg1EC4/Dsg2 Ehis, where swapping occurs at nine amino acids upstream of Dsg1EC4 on the amino side and at four amino acids downstream on the carboxyl side. E-tag and His-tag were added at the carboxy-terminus. These domain-swapped molecules were generated with an aim to facilitate conformational epitope mapping of pemphigus foliaceus sera.

Table 1 Polymerase chain reaction primers used for production of desmoglein (Dsg) 1/Dsg2 domain-swapped molecules

	Primers					
	Primers for Dsg2 EC domain amino-terminus		Primers for Dsg1 EC domain		Primers for Dsg2 EC domain carboxy-terminus	
Recombinant molecules	5′ primer	3′ primer	5′ primer	3′ primer	5′ primer	3′ primer
Dsg1EC1/Dsg2 Ehis	NA	NA	Primer 1	Primer 2	Primer 3	Primer 4
Dsg1EC2/Dsg2 Ehis	Primer 5	Primer 6	Primer 7	Primer 8	Primer 9	Primer 4
Dsg1EC3/Dsg2 Ehis	Primer 5	Primer 10	Primer 11	Primer 12	Primer 13	Primer 4
Dsg1EC4/Dsg2 Ehis	Primer 5	Primer 14	Primer 15	Primer 16	Primer 17	Primer 4
Dsg1EC5/Dsg2 Ehis	Primer 5	Primer 18	Primer 19	Primer 20	NA	NA

Primer 1, 5′-GCAGCCATGGACTGGAGTTTCTTCAGAGTA-3′; Primer 2, 5′-TTCGTTGTCATTTATATCCAAAACCCTGACTCT-3′; Primer 3, 5′- AGGGTTTTGGATATAAATGACAACGAACCAGTG-3′; Primer 4, 5′-CCGGTCGACGCCGACATAGGAGTCATGCTGTGCT-3′; Primer 5, 5′-CGGGGTACCATGGCGCGGAGCCCGGGACGCGCGTA-3′; Primer 6, 5′-CCCTGACTCTTAGCTCTAAGGGTTTCTCTACA-3′; Primer 7, 5′-ACCCTTAGAGCT AAGAGTCAGGGTTTTGGATATAAA-3′; Primer 8, 5′-CTTTATTTTCCATGTAAGGGATATTATCATTGAC-3′; Primer 9, 5′-CCCTTACATGGAAAATAAAGTGCTTGAAGGGAT-3′; Primer 10, 5′-AGGGATATTATCATTGACATCCAAAATACGAATCTG-3′; Primer 11, 5′-CGTAATTTGGATGTCAATGATAATATCCCTTACATGGA-3′; Primer 12, 5′-ATGAATGCCTTCTTTTACATTTAACACAGTCACAGAAAT-3′; Primer 13, 5′-CT GTGTTAAATGTAAAAGAAGGCATTCATTTTAAAAGC-3′; Primer 14, 5′-CAGAAATTGCAATGGGTGTAGGCTTGTATTTACT-3′; Primer 15, 5′-CCTACACCCATTGCAATTTCTGTGACTGTGTT-3′; Primer 16, 5′-ACAGTTGTCGTTGGTCGTCATTACCAAAACTTTGAA-3′; Primer 17, 5′-GGTAATGACGACCCCACACTGATAGAGCCTG-3′; Primer 18, 5′-GTCGTCATTACCGATGTCTTCAACATTGATAAG-3′; Primer 19, 5′-GTTGAAGACATCGGTAATGACGACAGGACTAAT-3′; Primer 20, 5′-GGAGTCGACATGTACATTGTCTGATAACAAATC-3′; EC, extracellular; NA, not applicable.

site for NcoI and SalI in the 5′ end (primers 1 and 5) and 3′ end (primers 4 and 20), respectively. The intervening primers were nucleotide sequences representing adjoining EC domains of Dsg1 and Dsg2. To illustrate the production of Dsg1EC2/Dsg2 domain-swapped molecules, a first-round PCR generated Dsg2EC1 and Dsg1EC2 products, using primers 5 and 6 on a template of pQE-hDsg2 and primers 7 and 8 on a template of pEVmod-PFIg. The two fragments were then annealed with each other and extended by mutually primed synthesis. Primers 5 and 8 were then added and a second-round PCR produced combined Dsg2EC1 and Dsg1EC2. Finally, we joined the carboxyl part, representing Dsg2EC3–5, in a similar way. A baculovirus transfer vector containing cDNA coding for E-tag and His-tag at the carboxyl end was obtained after removal of Dsg2 in pQE-hDsg2 by digestion with NcoI and XhoI. Dsg1EC2/Dsg2 PCR product was digested by NcoI and SalI, followed by ligation to the NcoI-XhoI cut-pQE-hDsg2. Five plasmids were produced and were named pQE-Dsg1EC1–5/Dsg2. Their sequences were verified before large-scale protein production.

Production of baculovirus protein

Recombinant proteins were produced as previously described.[7,11] In brief, the plasmids were cotransfected with Sapphire baculovirus DNA (Orbigen, San Diego, CA, U.S.A.) into cultured insect Sf9 cells. Recombinant virus was collected in the supernatant and was amplified by several rounds of passage in Sf9 cells. High-Five cells (Invitrogen, San Diego, CA, U.S.A.) cultured in serum-free EX Cell 405 medium (JRH Bioscience, Lenexa, KS, U.S.A.) were infected by baculovirus and incubated at 27 °C for 3–4 days. The baculoproteins were collected as supernatant after removal of cellular debris by centrifugation and were stored at −70 °C.

Immunoblotting of recombinant proteins

Recombinant proteins were fractionated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, U.S.A.). To visualize the protein, anti-E-tag mAb (Amersham Bioscience, Piscataway, NJ, U.S.A.) at 1 : 5000 dilution and alkaline phosphatase-conjugated anti-mouse IgG antibody (Zymed, San Francisco, CA, U.S.A.) at 1 : 4000 dilution were used as primary and secondary antibody, respectively. The concentrations of baculoproteins were adjusted by dilution with uninfected High-Five supernatant until all bands were of similar density on immunoblotting.

Competition enzyme-linked immunosorbent assay

Five domain-swapped baculoproteins, Dsg1EC1–5/Dsg2 Ehis, of similar density to Dsg1-His and Dsg2-His on immunoblotting, were concentrated fivefold by Centriprep (Millipore) and then mixed together in an equal volume ratio. Ten different PF sera which recognized more than one EC domain were selected. One microlitre of serum was mixed with 200 μL of recombinant proteins and incubated at room temperature for 30 min. This was then subjected to ELISA against the entire EC domains of Dsg1. When necessary, sera were diluted to keep an optical density (OD) below 1·2. The competition rate was calculated using the following formula: competition rate (%) = $[1 - (OD_{competitor} - OD_{positive})/(OD_{negative} - OD_{positive})] \times 100$; where $OD_{positive}$, $OD_{negative}$ and $OD_{competitor}$ were the ODs obtained when sera were incubated with Dsg1-His, uninfected High-Five supernatant and recombinant baculoproteins of interest, respectively. Culture supernatants of Dsg1-His and Dsg1EC1–5/Dsg2 contained sufficient baculoprotein to achieve the plateau competition level (data not shown).

Immunoprecipitation-immunoblotting analysis

Immunoprecipitation was carried out by mixing 10 μL bed volume of Protein G Sepharose 4 Fast Flow (GE Healthcare, Uppsala, Sweden), 250 μL of baculoproteins and 5 μL of either anti-E-tag mAb (Amersham Bioscience; diluted tenfold) or PF sera. The mixture was incubated at 4 °C overnight with rotation and then washed four times with Tris-buffered saline with 1 mmol L^{-1} $CaCl_2$ and 0·05% Tween 20. The immunoprecipitated proteins were then resuspended in SDS sample buffer, fractionated by SDS-PAGE and labelled by horseradish peroxidase-conjugated anti-E-tag antibody (GE Healthcare, Amersham, U.K.) diluted at 1 : 5000. They were visualized by using Western Lightning Chemiluminescence Reagent Plus (PerkinElmer LAS, Shelton, CT, U.S.A.) and autoradiography. Dsg1-His and Dsg2-His served as positive and negative controls of epitope profile analysis.

Results

Production of recombinant molecules in baculovirus expression system

Five recombinant molecules were constructed in the present study. For EC1–3 and EC5 Dsg1/Dsg2 domain-swapped molecules, amino acids adjoining adjacent EC domains of Dsg1 and Dsg2 changed exactly at the interface between swapped domains. For the EC4 counterpart, due to failure of generation of a similar construct after several trials, we designed another set of primers so that the final construct represented swapping at nine and four amino acids upstream and downstream of Dsg1EC4, respectively (Fig. 1). Domain-swapped molecules, together with Dsg1-His and Dsg2-His, were expressed in a baculovirus expression system. These baculoproteins were represented by doublet bands on immunoblotting (Fig. 2). Bands with higher molecular weight represented proproteins, whereas lower molecular weight bands represented mature forms after cleavage of prosequence. The presence of such doublets represented saturation of the proteolytic enzymes in insect cells.[4,6,10,14]

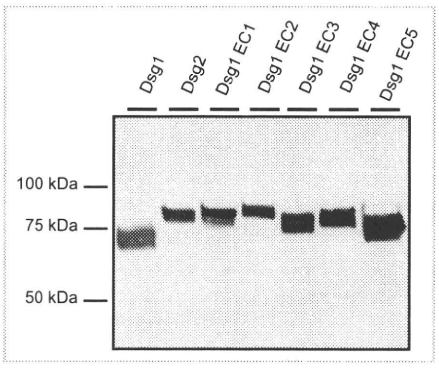

Fig 2. Production of recombinant molecules in baculovirus expression system. The molecules were visualized by immunoblotting with anti-E-tag monoclonal antibody. The intensity of each band was similar on immunoblotting before proceeding to immunoprecipitation-immunoblotting.

Competition enzyme-linked immunosorbent assay

The potential drawback of domain-swapped molecules was loss of epitopes formed by two or more domains in the original molecule. We estimated epitope loss in this set of domain-swapped molecules by competition ELISA. The competition rate of Dsg1EC1–5 mixture was 79 ± 20% (mean ± SD), indicating that about 80% of epitopes in Dsg1-His were represented. The corresponding value for Dsg2-His, negative control, was 4 ± 3%.

EC1 and EC2 were major epitopes of pemphigus foliaceus sera in active stage and in remission

Before epitope mapping, we confirmed that similar amounts of domain-swapped proteins, Dsg1-His and Dsg2-His were immunoprecipitated by anti-E-tag mAb (Fig. 3).

IP-IB analysis of epitopes of sera taken from 32 patients with PF in active disease showed a profile skewed towards the amino-terminus of Dsg1 (Fig. 3). These sera had a median Dsg1 ELISA index of 265 (interquartile range 181–837) and were taken from six, 19 and seven patients with mild, moderate and severe disease, respectively. Nineteen sera (59%) showed immunoreactivity to more than one EC domain whereas the rest (41%) recognized only one domain. The proportion of PF sera that recognized various Dsg1 EC domains was 88% (EC1), 50% (EC2), 13% (EC3), 22% (EC4) and 0% (EC5), respectively (Figs 3 and 4). EC1 and EC2 were thus the major epitopes of sera from patients with PF having active disease. Moreover, immunoreactivity to EC3 or EC4 (n = 9) often cosegregated with immunoreactivity to either EC1 or EC2 or both (eight of nine, 89%). Only one serum reacted exclusively with EC4 (11%).

We also analysed the epitope profile of sera from 19 patients with PF in remission who had a median Dsg1 ELISA index of 127 (interquartile range 92–405). Their remission duration ranged from 1 to 23 months. They were all receiving prednisolone 0·1–0·5 mg kg^{-1} daily with or without azathioprine 1–3 mg kg^{-1} daily, except for two patients who were on topical steroids only. Among them, three patients with PF had prolonged remission of longer than 18 months (two were receiving prednisolone 7 mg daily and one was not on any systemic treatment). Similar to PF sera in active disease stage, EC1 and EC2 were the major epitopes of PF sera from patients in remission (EC1, 84%; EC2, 53%; EC3, 11%; EC4, 21%; EC5, 0%; Fig. 4). All PF sera from patients in remission that reacted with either EC3 or EC4 also recognized EC1 or EC2.

Figure 5 summarizes the reactivity of all 91 sera (from 34 patients with PF) towards Dsg1 EC1–5 domains according to activity stages. In contrast to Figure 4, individual patients contributed different numbers of sera to each activity stage in Figure 5 and this could potentially confound the immunoreactivity percentage observed in various EC domains. Despite this

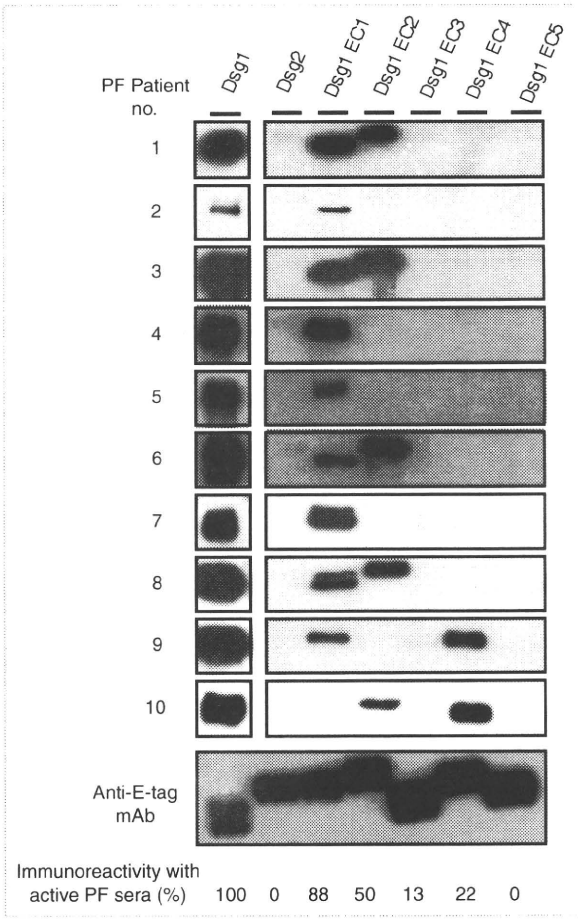

Fig 3. Representative immunoprecipitation-immunoblotting (IP-IB) epitope analysis of pemphigus foliaceus (PF) sera from 10 patients with active disease. When more than one active stage was present in an individual patient, the one that represented the most serious disease activity was illustrated in this figure. The lower panel illustrates that similar amounts of domain-swapped proteins Dsg1-His and Dsg2-His were immunoprecipitated by anti-E-tag monoclonal antibody (mAb). In IP-IB of individual PF sera, Dsg1-His and Dsg2-His served as positive and negative controls, respectively. The percentages (bottom) represent summary statistics of reactivity to various Dsg1 EC domains found in 32 patients with PF having active disease.

limitation, a predominant amino-terminal Dsg1 immunoreactivity was still observed across activity stages. No immunoreactivity to EC5 was noted in PF sera of either active or remission stage, although a few mucocutaneous PV sera did react with EC5 (data not shown).

Longitudinal follow-up epitope analysis in individual patients with pemphigus foliaceus showed that, in most cases, immunoreactivity to the amino-terminus of desmoglein 1 persisted even upon remission

We monitored the epitope profile of 21 patients with PF longitudinally. Seventeen patients obtained remission within the follow-up period. The median serum follow-up time was 16 months (range 2–106). Thirteen patients with PF (62%) were followed up for more than 1 year. In most patients with PF, Dsg1 ELISA index showed good correlation with disease activity and this correlation was better than that observed for IIF titre (Figs 6 and 7).

These patients could be classified into three groups, according to how their epitope profile varied with disease activity. In group 1, there was no change in epitope profile across activity stages or upon remission, despite some variations in band densities with time (Fig. 6a–c). This group comprised 13 patients whose sera recognized EC1 only (n = 4), EC1 + EC2 (n = 5), EC1 + EC4 (n = 2), EC1 + EC2 + EC4 (n = 1) and EC2 + EC4 (n = 1).

Group 2 comprised six patients whose sera recognized either EC1 + EC2 (n = 5) or EC1 + EC3 (n = 1). Among these patients, although there was complete loss/gain of EC2 or EC3 immunoreactivity across activity stages, their EC1 immunoreactivity remained persistent (Fig. 6d–f). In some cases, complete loss of EC2/3 reactivity occurred when disease activity was lower (Fig. 6e,f). In another case, gain in EC2 reactivity was associated with increasing severity initially, but later, upon remission, EC2 reactivity persisted, instead of vanishing (Fig. 6d). Thus, change in EC2/3 immunoreactivity did not correlate well with disease activity, unlike Dsg1 ELISA index. For groups 1 and 2 (n = 19 of 21, 90%), immunoreactivity to the amino-terminus of Dsg1 was maintained across activity stages, even upon remission.

Group 3 included two patients in whom immunoreactivity to EC1 was lost upon remission (n = 2, 10%). One patient had an epitope localized in EC1 when PF was more active, but immunoreactivity to EC1 was lost upon remission and changed to EC2 + EC3. The change in epitope profile was maintained early in the relapse course and the morphology of the patient's skin lesions was similar at different time points when he had different epitope profiles (Fig. 6g). Another patient recognized EC1 + EC2 when his PF was active, but reactivity to EC1 was lost upon remission, leaving only EC2 (Fig. 6h). These two patients lost their EC1 reactivity early in the first month of remission.

The 17 remission sera of the longitudinal study included patients having a remission duration of 1–23 months. This enabled us to observe the stability of epitope profile at different time intervals after remission. Three of the patients in remission had a prolonged remission duration (> 18 months) and were on maintenance treatment with prednisolone 7 mg daily or no systemic therapy. They were representative of patients having sustained clinical remission and their epitope profile remained unchanged (Fig. 7). For other patients having a shorter remission duration, most retained Dsg1 amino-terminal reactivity even upon remission, except for two patients (group 3 cases, Fig. 6g,h). We were unable to identify any clinical or therapeutic parameters, including remission duration, related to the loss of Dsg1 amino-terminal reactivity on remission.

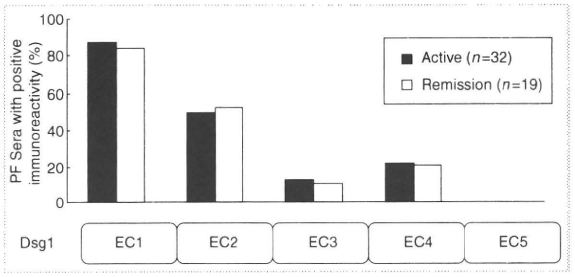

Fig 4. Comparison of desmoglein (Dsg) 1 epitope profile between pemphigus foliaceus (PF) sera taken at active and remission stages. The distribution of epitopes in active and remission stages, from 32 and 19 patients with PF, respectively, is shown. Each individual contributed only one serum to active or remission stages for calculation of immunoreactivity percentage in this figure. For individuals having more than one active serum, the one which represented the most serious disease activity was chosen; conversely, for patients having more than one remission serum, the one with the lowest Dsg1 enzyme-linked immunosorbent assay index was chosen. Immunoreactivity towards the amino-terminus of Dsg1 was prevalent in both active and remission stages with EC1 (84–88%) and EC2 (50–53%) being the major epitopes. EC5 immunoreactivity was consistently absent in all active or remission sera.

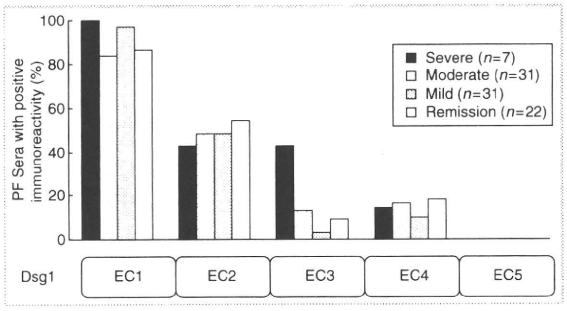

Fig 5. Summary of desmoglein (Dsg) 1 epitope profile of all 91 sera in 34 patients with pemphigus foliaceus (PF) according to activity stages subdivided into severe (n = 7), moderate (n = 31), mild (n = 31) and remission (n = 22) stages. Individual patients contributed different numbers of sera to each activity stage for calculation of immunoreactivity percentage in this figure. A predominant amino-terminal Dsg1 immunoreactivity was observed across activity stages, even upon remission. PF sera did not show any immunoreactivity to EC5 in all activity stages.

Discussion

Recombinant domain-swapped molecules have been employed in characterizing functional domains of molecules and antibody epitopes in various autoimmune diseases.[6] By domain-swapping between analogous molecules, conformational epitopes on the native molecule are mostly preserved and are theoretically better than truncated peptides which mainly represent linear epitopes. We have chosen Dsg2 as a backbone for our domain-swapped molecules because of its similarity with Dsg1 in terms of amino acid sequence and arrangement of EC domains, together with its nonreactivity to pemphigus sera.[4,17] This new set of domain-swapped molecules has several merits over the previous versions using Dsg1 and Dsg3.[6,9,10] Firstly, the new design allows direct precise conformational epitope mapping according to EC structure of Dsg1. Secondly, although antibodies to Dsg3 and Dsg1 do not show cross-reactivity most of the time,[14] recent findings show the presence of Dsg1/Dsg3 cross-reactive monoclonal scFv from a patient with PF.[13] Sera from patients with mucocutaneous PV have both anti-Dsg1 and anti-Dsg3 antibodies. Moreover, nonpathogenic anti-Dsg3 antibodies were reported in patients having either nonendemic PF (2·8%) or endemic PF (7·5–43%).[18,19] In the presence of cross-reactive antibodies or when serum samples contain both anti-Dsg1 and anti-Dsg3 antibodies, the use of Dsg1/Dsg2 domain-swapped molecules for epitope mapping may be more appropriate as immunoreactivity towards the Dsg1 and Dsg3 portion of chimeric molecules cannot easily be distinguished by IP-IB analysis.

However, an inherent problem of using any domain-swapped molecules is that after domain swapping, any epitopes represented by two or more domains in the original molecule may be lost. These epitopes can either be linear (represented by adjacent amino acids on adjoining domains) or conformational (represented by amino acids on distant domains but brought together in close proximity after peptide folding). In this study, it is essential to estimate epitope loss as we have swapped five domains in one molecule. Our results show that 80% of the epitopes in Dsg1 molecules are represented by the five domain-swapped molecules.

This study shows an outstandingly high prevalence of EC1 immunoreactivity in PF sera and this domain is of utmost importance in mediating adhesion. Ultrastructural crystal analysis of C-cadherin ectodomain suggested the critical involvement of a twofold symmetrical 'strand dimer interface' in EC1 domains of opposing molecules for the *trans* (intercellular) adhesive function of classical cadherins.[20] More recently, three-dimensional reconstruction of desmosomal cadherin, which involved the computational fitting of C-cadherin X-ray structure into data obtained both in mouse epidermis by electron tomography and in human epidermis by cryo-electron tomography of vitreous sections, suggested that classical and desmosomal cadherins use an analogous adhesive interface involving EC1 domains.[21,22] Furthermore, studies in adoptive PV mice generated AK23, a highly pathogenic mAb, which recognized an epitope located in the predicted 'strand dimer interface' in EC1.[11] In E-cadherin, the presence of a small fragment of prosequence near EC1 could inactivate adhesive function by steric hindrance.[23] Taken together, this suggests that direct inhibition of intercellular adhesion by antibodies is important for blister formation. Although antibodies towards EC2–4 only were seen less frequently in our study, they may also play a role in acantholysis as synergistic pathogenicity of mAbs has been demonstrated by ascites formation assay in mice.[24]

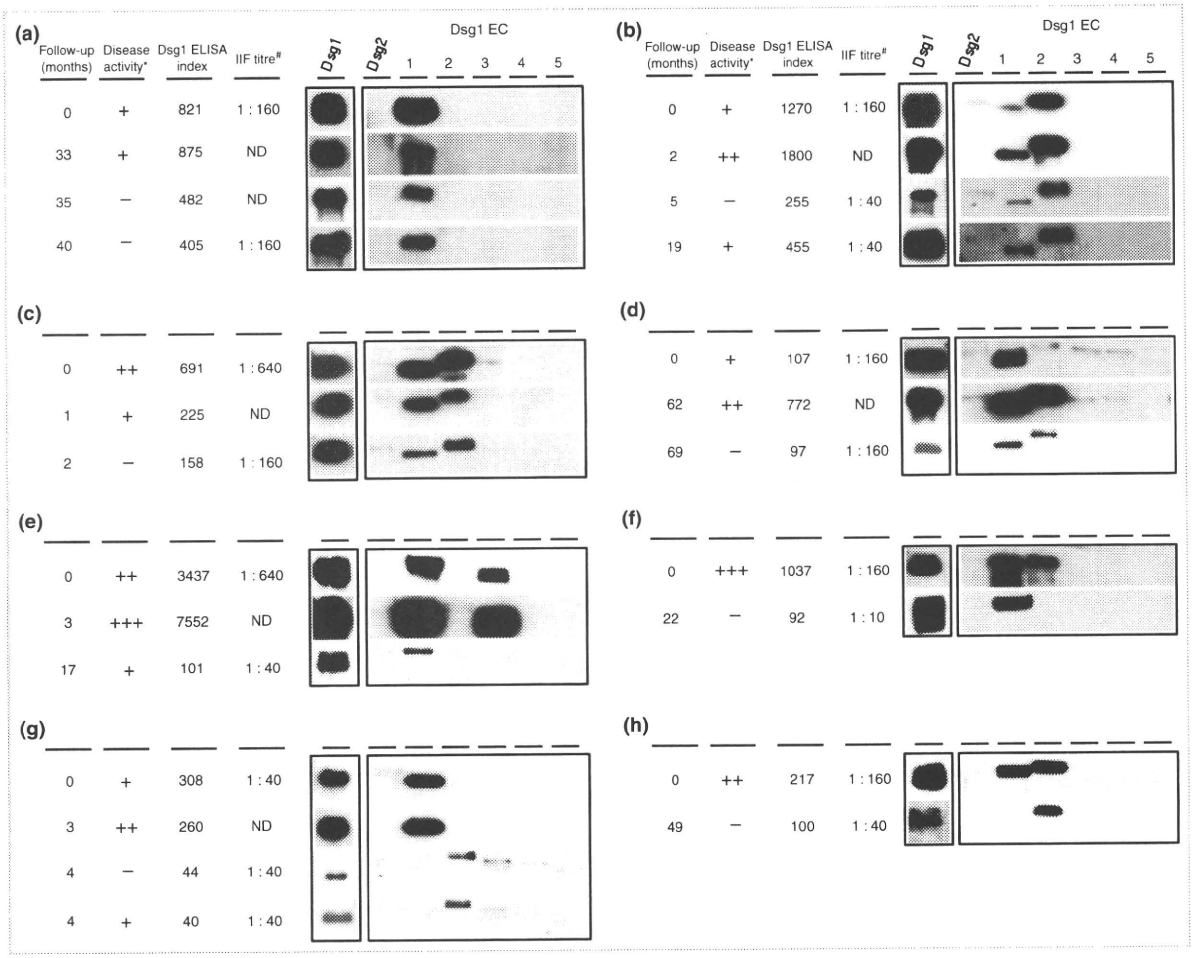

Fig 6. Representative results showing variation of desmoglein (Dsg) 1 enzyme-linked immunosorbent assay (ELISA) indexes and Dsg1 epitope profile across activity stages in individual patients with pemphigus foliaceus (PF). For each patient with PF, indirect immunofluorescence (IIF) titres of at least two sera in different activity stages are also shown. Regarding variation of epitope profile on longitudinal follow-up of 21 patients, three patterns were identified. These patterns are illustrated by representative data from eight patients with PF. For group 1, no change in epitope profile could be observed (a–c). For group 2, gain/loss of immunoreactivity to EC2/3 occurred across activity stages but immunoreactivity to EC1 remained persistent (d–f). For group 3, immunoreactivity to EC1 was lost upon remission and EC2 ± EC3 became the major epitope(s) when disease activity became lower (g,h). Immunoreactivity to EC5 was not found in any remission sera. In these patients, correlation of disease activity was best observed when Dsg1 ELISA was used as a measure of antibody titre, compared with IIF test. *+++, ++, + and − represent severe, moderate, mild and remission stage, respectively. #IIF, indirect immunofluorescence; ND, not done.

In endemic PF, a previous study showed restricted EC5 reactivity in remission or preclinical sera and development of EC1 and/or EC2 reactivity in active disease, indicating that intramolecular epitope spreading might modulate disease relapse or remission.[10] However, the current study in nonendemic PF shows that EC1 and EC2 remain as the major epitopes in both active and remission sera (Fig. 4). In follow-up study of 21 patients, Dsg1 amino-terminal immunoreactivity was retained in all cases except for two in whom, upon remission, antibody epitopes spread from EC1 to EC2 ± 3, but never to the carboxy-terminus (Fig. 6). Therefore, despite both titre and epitopes being important determining factors of antibody pathogenicity,[10,11,14,15,24,25] in most patients with nonendemic PF only changes in titre correlate well with disease activity.

Endemic PF shares similar clinical and histopathological features with nonendemic PF; none the less, it has its own unique epidemiological characteristics such as occurrence in rural areas, clustering of cases along rivers and in the same family and association with bloodborne diseases transmitted by arthropods.[2,26] Endemic PF also has some unique immunological features such as the presence of anti-Dsg1 antibodies in healthy neighbourhood inhabitants[27] and a distinctive anti-Dsg1 IgM response.[28] Thus, although Dsg1 is the common antigen in endemic and nonendemic PF,[29] we speculate that there are substantial differences in the course leading to Dsg1

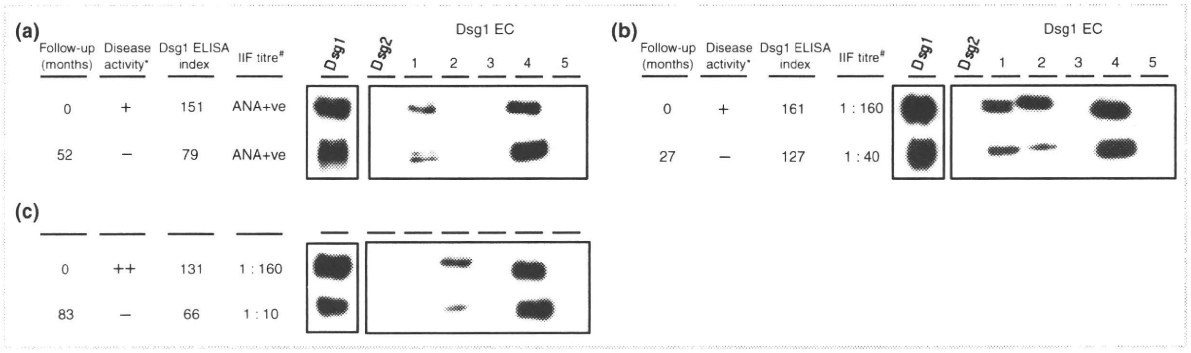

Fig 7. Desmoglein (Dsg) 1 epitope profile of three patients with pemphigus foliaceus (PF) having the longest remission duration in this study. Their epitope profile remained unchanged even after prolonged remission and thus, they belonged to group 1 patients illustrated in Figure 6. The remission sera of patients (a)–(c) were taken at 19, 22 and 23 months postremission, respectively. At that time, patients (b) and (c) were on maintenance treatment with prednisolone 7 mg daily, whereas patient (a) was not on any systemic treatment. *++, + and – represent moderate, mild and remission stage, respectively. #IIF, indirect immunofluorescence; ANA+ve, titre cannot be determined because of interference by positive antinuclear antibody; ELISA, enzyme-linked immunosorbent assay.

autoimmunity development so that findings in one form of PF may not be directly extrapolated to another.

In conclusion, this study is the first that comprehensively delineates the epitope profile of nonendemic PF according to EC structure of Dsg1 and has demonstrated the predominant amino-terminal immunoreactivity towards Dsg1 in these patients, even across activity stages. We have constructed a new set of domain-swapped molecules that can be employed for making epitope-specific ELISA in future. This is potentially useful for diagnosis or disease monitoring in endemic PF in which a high prevalence of anti-Dsg1 antibodies is found even in healthy inhabitants, but with reactivity restricted to EC5, and the development of active disease being marked by epitope spreading to EC1 and EC2.[10,19,27] There are some situations in which the Dsg compensation theory seems not to be valid, like the absence of mucosal involvement in cutaneous PV[30] and the presence of anti-Dsg3 antibodies in PF.[18,19] Moreover, despite similar immunoreactivities to Dsg as in classical pemphigus, morphological variants, like pustular PF or pemphigus herpetiformis, do exist.[31,32] Epitope mapping, using domain-swapped molecules, may shed some light on the pathophysiological basis in these scenarios.

What's already known about this topic?

In endemic pemphigus foliaceus (PF), it was previously shown that desmoglein (Dsg) 1 autoimmunity evolved from nonpathogenic, towards extracellular domain (EC) 5, to pathogenic, towards EC1–2; furthermore, immunoreactivity shifted back to EC5 upon remission. It is not known whether a similar phenomenon occurs in nonendemic PF.

What does this study add?

In nonendemic PF, loss of Dsg1 amino-terminal reactivity occurs only rarely upon remission. In rare circumstances when epitope spread does occur, the pattern is very different from endemic PF: the epitope spreads to EC2 ± EC3, but never to EC5. Only Dsg1 ELISA index, but not change in epitope profile, correlates well with disease activity in nonendemic PF.

Acknowledgments

We thank Mrs Minae Suzuki for performing the ELISA and indirect immunofluorescence test and Mrs Yoshiko Fujii for helping in the preparation of recombinant proteins. This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; the Health and Labour Sciences Research Grants for Research on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan and Keio Gijuku Academic Development Funds.

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doi:10.1016/j.jaad.2010.01.028

Antidesmocollin-1 antibody–positive, antidesmoglein antibody–negative pemphigus herpetiformis

To the Editor: Pemphigus is an autoimmune skin disease that is divided into two major subtypes—pemphigus vulgaris (PV) and pemphigus foliaceus (PF).[1] Pemphigus herpetiformis (PH) is recognized as a distinct variant of pemphigus by its pruritus, rarity of mucosal involvement, histologically eosinophilic spongiosis, and good response to sulfones. Clinical characteristics of PH include erythematous papules and plaques with occasional vesicles in a herpetiform arrangement.[2] The major target antigens of PH are desmogleins (Dsg) 1 and 3, the targets for PF and PV, respectively.[2] We present a case of PH with immunoglobulin G (IgG) autoantibodies to desmocollin (Dsc) 1 but without antibodies to Dsgs.

An 83-year-old man was referred to our hospital because of an exudative bulla and erosion on the foot and annular, erythematous plaque on the trunk (Fig 1). Laboratory tests revealed mild leukocytosis and marked eosinophilia. A biopsy specimen from an erythematous plaque revealed intraepidermal eosinophilic pustules without acantholysis and mild spongiosis with eosinophilic infiltration (Fig 2, *A*). Direct immunofluorescence for IgG was positive on cell membranes in the upper half of the epidermis (Fig 2, *B*). Indirect immunofluorescence using normal human skin sections revealed IgG anti–cell membrane antibodies (Fig 2, *C*). An enzyme-linked immunosorbent assay revealed that the indices of antibodies to both Dsg1 and Dsg3 were below 5. We performed immunoblot analysis for normal human epidermal extract using SuperSignal West Dura Extended Duration Substrate as a substrate for horseradish peroxidase. Anti-Dsc 1 and 3 monoclonal antibodies (Progen, Heidelberg, Germany) and PV sera (reported previously)[3] were used as controls. On this analysis, IgG antibodies of the patient's serum showed doublet protein bands (Fig 2, *D*, lane 3), which were perfectly matched to the bands reacting with the anti-Dsc1 monoclonal antibody (Fig 2, *D*, lane 2), but were different from those reacting with anti-Dsc3 (Fig 2, *D*, lane 1), Dsg1 or Dsg3 monoclonal antibodies (Fig2, *D*, lane 4). We diagnosed this case as PH with an atypical serum antibody pattern; anti-Dsc1 antibody positive without anti-Dsgs antibodies. Treatment with prednisolone 30 mg, diaminodiphenyl sulfone 75 mg, and azathioprine 50 mg for 3 months resolved the cutaneous lesions without exacerbation.

We have reported a case of PH with autoantibodies to Dsc1, a target in IgA pemphigus. It is well known that there is some degree of overlap between the various types of pemphigus and their associated

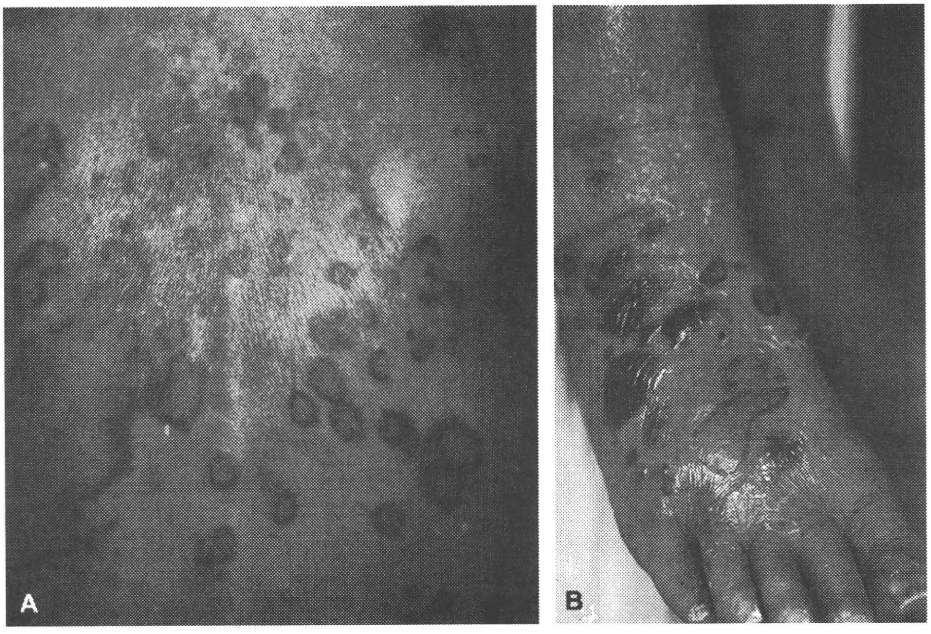

Fig 1. A, Annular or gyrate erythema with elevated borders on the back. **B**, Blood-filled bulla and erosion on the dorsal surface of the right foot.

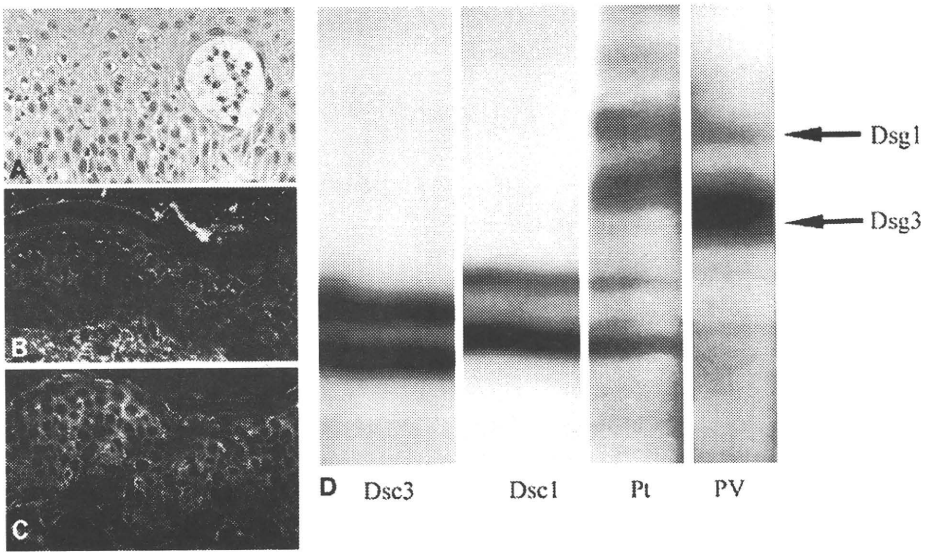

Fig 2. **A**, Histology of a perilesional biopsy specimen revealed an intraepidermal bulla filled with eosinophils but without acantholysis. Mild spongiosis and scattered eosinophils are also seen. **B**, Direct immunofluorescence revealed immunoglobulin G (IgG) cell surface deposits in the upper half of the epidermis. **C**, Indirect immunofluorescence revealed IgG anti–cell surface antibodies reactive with the granular layer and upper two thirds of the spinous layer of the epidermis. **D**, Immunoblotting of normal human epidermal extracts revealed that IgG antibodies of the patient's serum reacted with the 111-kDa "a" form of Dsc1 and the 103-kDa "b" form of Dsc1 (lane 3), which were also detected by control anti-Dsc1 monoclonal antibody (lane 2). Control anti-Dsc3 monoclonal antibody reacted with the 109-kDa "a" form of Dsc3 and the 100-kDa "b" form of Dsc3 (lane 1). Control pemphigus vulgaris serum reacted with the 160-kDa pemphigus foliaceous antigen (Dsg1) and with the 130-kDa pemphigus vulgaris antigen (Dsg3). (**A**, Hematoxylin–eosin stain; original magnifications: **A**, ×400; **C**, ×400.)

antigens. For example, patients with PV sometimes develop antibodies to the primary PF antigen, Dsg1.[1] Similarly, patients with paraneoplastic pemphigus are known to have antibodies to Dsg3 in addition to envoplakin and periplakin antibodies.[4] A case of PH with anti-Dsg1 and anti-Dsc3 antibodies has been reported.[5] However, to the best of our knowledge, this is the first case of PH without anti-Dsg antibodies.

The pathogenesis of PH is not known, but the antibodies in PH are believed to cause inflammation through complement fixation and cytokine release without cell detachment of keratinocytes, although the antibodies in PV or PF can cause the latter.[2] Therefore, in PH, there is less acantholysis than in PV or PF.[2] We speculate that the pathogenesis in our patient follows this theory, although the pattern of autoantibodies was different.

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Funding sources: None.

Conflicts of interest: None declared.

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doi:10.1016/j.jaad.2009.09.016

Atypical methotrexate-induced ultraviolet recall phenomenon during the treatment of ectopic pregnancy

To the Editor: A 26-year-old white female developed a painful rash on her chest and abdomen 12 hours after administration of a single intramuscular dose of methotrexate 50 mg for the treatment of an ectopic pregnancy. She described the dermatitis as being "like a sunburn," and the dermatitis was exquisitely tender to touch. She denied radiation exposure from a radiographic procedure within the past 5 years or radiation therapy for malignancy. She could not recall having had a significant sunburn, although she had a lifetime history of excessive sun exposure. She had never been treated with methotrexate and was taking no other medication. There was no significant medical history, and she was otherwise healthy.

The physical examination revealed a large, well demarcated, brightly erythematous to violaceous patch encompassing the chest and epigastric areas (Fig 1). She demonstrated significant discomfort, with even mild palpation of the affected area. The rash spared the extensor surfaces and the intertriginous and acral areas. A biopsy specimen obtained from the area of dermatitis revealed an interface dermatitis with keratinocyte balloon degeneration and atypia (Fig 2). Spontaneous resolution occurred within approximately 10 days without sequelae. The differential diagnosis included toxic erythema of chemotherapy (TEC) and a variant of radiation recall dermatitis (RRD) referred to as the ultraviolet recall phenomenon (URP).

The clinical features of TEC include erythematous patches or plaques with a petechial or dusky appearance affecting the acral, intertriginous, and extensor surfaces.[1] The histopathology of TEC may reveal eccrine syringometaplasia, epidermal dysmaturation, keratinocyte necrosis/apoptosis, and vacuolar degeneration.[1] The intramuscular administration of methotrexate for termination of an ectopic pregnancy has been reported to cause an acral TEC.[2]

Radiation recall dermatitis is characterized by an inflammatory reaction in an area of previously irradiated skin, after administration of specific medications.[3] The most common precipitating agents are cytotoxic medications, such as actinomycin D, docetaxel, 5-fluorouracil, and methotrexate. RRD is most often seen in patients undergoing chemotherapy in conjunction with radiation for an underlying malignancy. The chemotherapeutic doses of methotrexate vary between 200 and 400 mg/kg.[3] A similar phenomenon has occurred in response to ultraviolet exposure in the presence of the same cytotoxic medications.[4,5] URP differs from RRD in that the initial sunburn or episodes of sun exposure may go unnoticed by the patient, and can occur in areas of

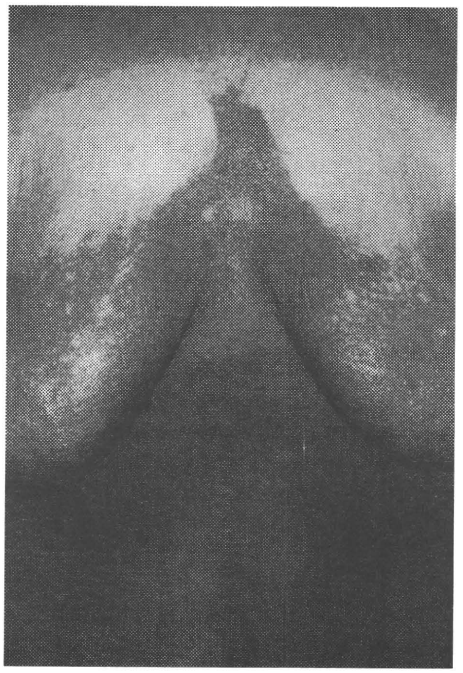

Fig 1. Erythematous to violaceous patch on the chest and abdomen after intramuscular administration of methotrexate for the management of an ectopic pregnancy.

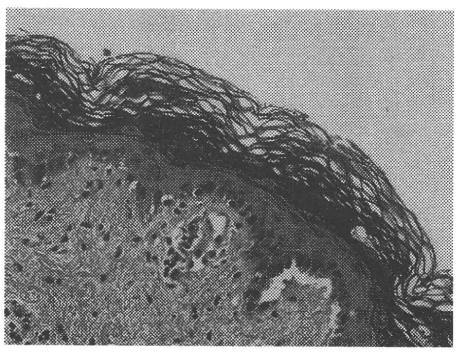

Fig 2. Mild interface dermatitis with keratinocyte ballooning and atypia. (Hematoxylin–eosin stain; original magnification: ×40.) Digital image provided by the University of Pittsburgh Dermatopathology Unit.

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The Protease Inhibitor Alpha-2-Macroglobuline-Like-1 Is the p170 Antigen Recognized by Paraneoplastic Pemphigus Autoantibodies in Human

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Abstract

Background: Paraneoplastic pemphigus (PNP) is a devastating autoimmune blistering disease, involving mucocutaneous and internal organs, and associated with underlying neoplasms. PNP is characterized by the production of autoantibodies targeting proteins of the plakin and cadherin families involved in maintenance of cell architecture and tissue cohesion. Nevertheless, the identity of an antigen of Mr 170,000 (p170), thought to be critical in PNP pathogenesis, has remained unknown.

Methodology/Principal Findings: Using an immunoprecipitation and mass spectrometry based approach, we identified p170 as alpha-2-macroglobuline-like-1, a broad range protease inhibitor expressed in stratified epithelia and other tissues damaged in the PNP disease course. We demonstrate that 10 PNP sera recognize alpha-2-macroglobuline-like-1 (A2ML1), while none of the control sera obtained from patients with bullous pemphigoid, pemphigus vulgaris, pemphigus foliaceus and normal subjects does.

Conclusions/Significance: Our study unravels a broad range protease inhibitor as a new class of target antigens in a paraneoplastic autoimmune multiorgan syndrome and opens a new challenging investigation avenue for a better understanding of PNP pathogenesis.

Citation: Schepens I, Jaunin F, Begre N, Läderach U, Marcus K, et al. (2010) The Protease Inhibitor Alpha-2-Macroglobuline-Like-1 Is the p170 Antigen Recognized by Paraneoplastic Pemphigus Autoantibodies in Human. PLoS ONE 5(8): e12250. doi:10.1371/journal.pone.0012250

Editor: H. Peter Soyer, The University of Queensland, Australia

Received June 2, 2010; **Accepted** July 24, 2010; **Published** August 18, 2010

Funding: This project was supported by Coordination Theme 1 (Health) of the European Community's FP7, Grant agreement number HEALTH-F2-2008-200515 (http://ec.europa.eu/research/health/medical-research/rare-diseases/projects/pemphigus_en.html)(to L.B.) and the Swiss National Foundation for Scientific Research (FNS/SNF 31003A-121966 and 31003A-09811, to L.B.)(http://www.projectdb.snf.ch/WebForms/Frameset.aspx). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Paraneoplatic pemphigus (PNP) is an autoimmune multiorgan syndrome associated with an underlying neoplasia [1,2,3]. Associated neoplasms include non-Hodgkin's lymphoma, chronic lymphatic leukaemia, Castleman disease, thymoma, and poorly differentiated sarcomas. PNP patients characteristically develop a severe polymorphous mucocutaneous eruption, features of which resemble pemphigus vulgaris, erythema multiforme, Stevens-Johnson syndrome and/or lichen. Involvement of internal organs, such as pulmonary and gastrointestinal tracts, is also observed [2,3,4]. Histologically, skin and mucosal lesions typically present intraepithelial cleavage, suprabasal acantholysis, and interface changes with necrotic and apoptotic keratinocytes [1,2,3,5,6]. Direct immunofluorescence (IF) microscopy studies disclose intraepidermal and/or basement membrane zone deposition of IgG and/or C3 complement component, whereas by indirect IF microscopy PNP sera contain autoantibodies binding to stratified, complex and simple epithelia, as well as to the myocardium [1,2,3]. PNP patients' autoantibodies typically bind to variable proteins including the plakin family members desmoplakin I and II, envoplakin, periplakin, plectin, and the bullous pemphigoid antigen 230 (BP230, also termed BPAG1-e). Furthermore, in analogy to pemphigus, desmoglein (Dsg) 1 and Dsg 3 are also consistently recognized [1,7,8,9]. Finally, PNP autoantibodies immunoprecipitate an unidentified protein of Mr170,000 (p170) from keratinocyte extracts [1,7,8,9,10,11].

The pathological mechanisms underlying tissue damage in PNP remain unclear. Different pathways have been involved to explain the polymorphous clinical features and multiorgan involvement [3]. Ample evidence indicates that autoantibodies against the desmosomal components Dsg 1 and Dsg3, which are expressed in stratified epithelia, play a central role in disrupting cell-cell adhesion of keratinocytes leading to acantholysis and intraepidermal blistering [9]. The vast majority of PNP sera contain autoantibodies directed against members of the plakin family. These proteins serve as versative cytolinkers connecting the intermediate filament cytoskeleton to distinct membrane sites,

such as desmososomes and hemidesmosomes. However, despite their ubiquitous expression, their role in disease initiation is unlikely, since they are cytoplasmic proteins [8,11]. Furthermore, though the majority of PNP sera also immunoprecipitate the p170 autoantigen, the search for the identity of this protein has proved to be technically challenging [9,12,13]. Finally, CD8+ cytotoxic T lymphocytes and other mononuclear cells are likely to contribute to tissue damage resulting in keratinocyte necrosis and apoptosis with a graft-versus-host disease-like phenotype in the skin [6,14].

Since identification of the target antigens is critical for a better understanding of the pathophysiology of a devastating multiorgan autoimmune syndrome such as PNP, we sought to characterize p170 by using a combination of immunoprecipitation and mass spectrometry analyses. We have identified A2ML1, a broad range protease inhibitor expressed in the epidermis and other tissues [15], as a novel autoantigen targeted by PNP autoantibodies.

Results

Analysis by MALDI-MS of p170 immunoprecipitated by a PNP serum

We first performed a preparative immunoprecipitation of PNP antigens from unlabelled cultured primary keratinocytes, differentiated for 5 days using a previously well characterized PNP serum sample [10]. Immunoprecipitated proteins were separated by 1D-SDS-PAGE and stained by Coomassie blue. The stained protein band migrating at Mr 170,000 was excised and subjected to MALDI-MS analysis. Mass profiles of the tryptic peptides are shown in **Figure S1** and the mono-isotopic masses derived from these profiles were used for the search in databases. We analyzed the data using the Mascot program (http://matrixscience.com) [16] to match the peptide mass fingerprint to two databases, NCBI and MSDB. When blasted against the NCBI database, the top score protein was alpha-2-macroglobuline-like-1 (A2ML1) (homo sapiens), with a calculated molecular mass of 161 kDa (gi|74271845, A8K2U0, Genbank accession No: AL832139). A total of 43 out of 128 peptides from trypsinized p170 matched the theoretical mass values of A2ML1 tryptic peptides. The 43 p170-tryptic peptides covered 43% of the A2ML1 sequence with an equal repartition of matches along the entire polypeptide. When blasted against the MSDB database, the top score was obtained for CAD48670, a protein of 165 kDa (sequence 1, homo sapiens, covered by patent WO0229058). There were 44 p170-tryptic peptides matching the theoretical peptide mass of trypsinized CAD48670, covering 43% of the whole sequence. CAD48670 represents a putative splice variant of A2ML1. Finding A2ML1 (A8K2U0 and CAD48670) in two different databases prompted us to hypothesize that p170 could be A2ML1.

Screening of PNP serum samples positive for p170

As the MALDI-MS results were obtained from p170 immunoprecipiated by a single PNP serum, which was consumed for the preparative immunoprecipitation, we first tested additional PNP serum samples to further characterize p170. Sera obtained from 20 PNP patients were screened by conventional immunoprecipitation using biosynthetically radiolabeled extracts from cultured differentiated human keratinocytes. Analysis of reduced immunoprecipitation samples showed that most PNP sera targeted proteins of Mr 250,000, 230,000, 210,000, and 190,000 corresponding to desmoplakin I, BPAG1-e/BP230, desmoplakin II and/or envoplakin, and periplakin, respectively as described [3]. Furthermore, 10 out of the 20 PNP serum samples also immunoprecipitated p170 (**Figure 1A, serum 3 to 12**). In our experiments, p170 displayed a Mr slightly higher than previously observed, above the 175,000 marker. This is most likely due to the use of different, prestained molecular weight markers. The stoichiometry of the precipitated proteins varied among the various PNP sera. In contrast, two serum samples obtained from normal volunteers (**Figure 1A** serum N1 and N2) did not significantly immunoprecipitate any proteins.

p170 is immunoprecipitated by PNP sera from the culture medium of keratinocytes

Since A2ML1 is a secreted protein [15], we next assessed whether p170 could be detected by immunoprecipitation with PNP sera from the culture medium of radiolabeled differentiated keratinocytes. Analysis of reduced immunoprecipitation samples showed that PNP sera reactive against p170 from keratinocyte

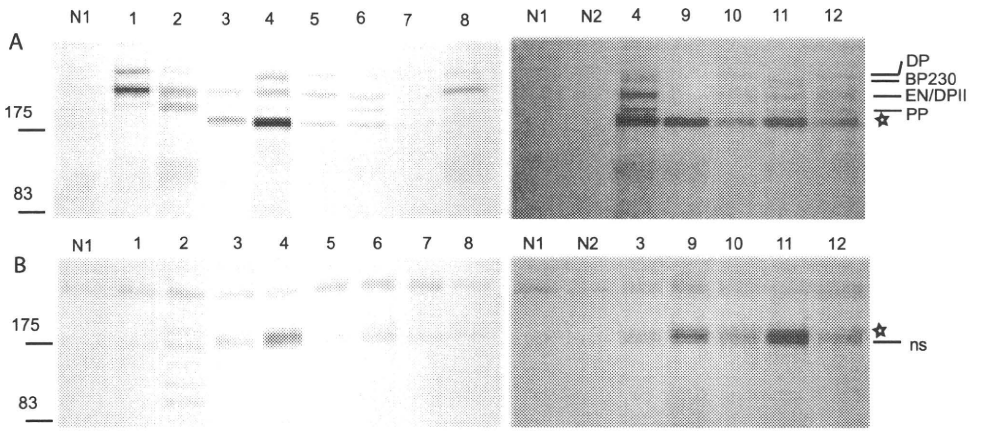

Figure 1. Analysis of keratinocyte proteins immunoprecipitated by PNP auto-antibodies. Normal sera (N1 and N2) or PNP sera (1–12) were used to immunoprecipitate proteins from radiolabelled keratinocyte extracts (A) or culture medium (B). Immunoprecipitates were denatured under reducing conditions, separated on 6% SDS-PAGE, and autoradiographed. p170 migration level is indicated by a star. PNP sera 1 and 2 immunoprecipitated negligible amounts of p170. BP230, desmoplakin (DPI, DPII), envoplakin (EN) and periplakin (PP) migration levels are indicated in panel A. In panel B, the non specific band also recognized by the control sera (N1, N2) with a slightly faster electrophoretic migration than p170 is indicated (ns).
doi:10.1371/journal.pone.0012250.g001

extracts (sera 3 to 12) also recognized a 170,000 protein from the culture medium of keratinocytes (**Figure 1B**). The relative intensity of the bands recognized by the various PNP sera from culture media corresponded to that observed using keratinocyte extracts. These results indicate that p170, like A2ML1, is a secreted protein.

Recognition of p170 by PNP sera is sensitive to reducing agents

So far, p170 has been exclusively detected by immunoprecipitation experiments, whereas immunoblotting studies using PNP sera invariably failed to detect p170 [7]. A2ML1 is a cysteine-rich protein with a predicted complex disulfide pattern [15], suggesting that A2ML1 possesses a very constrained structure. We therefore tested whether reducing conditions could play a role in the recognition of p170 by PNP sera by Western blotting. p170-positive PNP sera detected a protein of 170,000 from keratinocyte extracts under non-reducing conditions, but not reducing conditions (**Figure 2A**). The 170,000 protein was expressed more strongly by differentiated than by undifferentiated keratinocytes. This observation is in line with the reported increased expression of A2ML1 in the granular cell layers of the epidermis [15]. Interestingly, the reactive protein band showed the same electrophoretic migration of that recognized by the anti-A2ML1 antibody. The latter, however, bound to A2ML1 under both reducing and non-reducing conditions (**Figure 2B**). p170-negative PNP sera did not detect any 170,000 protein band under either reducing or non-reducing conditions (**Figure 2C**). These results suggest that PNP auto-antibodies recognize mainly conformation-dependent epitopes, which are lost under reducing denaturing conditions, explaining the lack of reactivity of PNP sera with p170 by Western blot analysis.

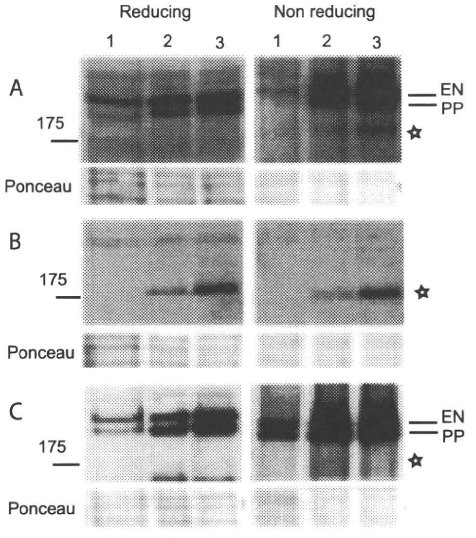

Figure 2. Denaturation conditions affect the recognition of p170 by PNP auto-antibodies by immunoblotting. Extracts (20 µg protein) from undifferentiated keratinocytes (lane 1), or differentiated for 4 days (lane 2) and 8 days (lane 3) were denatured in protein sample buffer containing or not 2-mercaptoethanol, separated on 8% SDS-PAGE and transferred onto nitrocellulose membrane for Western blot analysis using PNP serum 3 (A), anti-A2ML1 antibody (B), and PNP serum 2 (C). Sera numbering corresponds to that of Figure 1. Ponceau staining of the membranes is indicated as loading control. p170 migration level is indicated by a star. Envoplakin (EN) and periplakin (PP) migration levels are indicated.
doi:10.1371/journal.pone.0012250.g002

Anti-A2ML1 antibodies recognize p170 immunoprecipitated by PNP sera

The potential identity of p170 with A2ML1 was next analysed by immunoblotting of the radioactive PNP immunoprecipitates from keratinocyte extracts using anti-A2ML1 antibodies. A protein migrating exactly at the same level as S^{35}-labelled p170 was recognized by the anti-A2ML1 antibody. A good correlation between the relative ratio of the signals obtained by Western blotting using the anti-A2ML1 antibodies and those of the autoradiogram (n = 10) was observed (**Figure 3**). In contrast, when immunoprecipitates obtained either with p170-negative PNP sera or normal sera were tested, no signal was detected using anti-A2ML1 antibodies (**Figure 3**). These results strongly suggest that p170 is A2ML1.

A2ML1 expressed in transfected HEK 293T cells is recognized by p170-positive PNP sera

To further confirm the identity of p170 with A2ML1, we generated a cDNA construct for eukaryotic expression of a c-myc-tagged recombinant A2ML1 for both immunofluorescence and immunoprecipitation studies. First, we transfected Human Embryonic Kidney cells HEK 293T (American Type Culture Collection) and carried out double immunofluorescence microscopy studies using the PNP sera (serum 1 to 12) and an anti-c-myc antibody (representative sera are shown in **Figure S2**). The results showed that p170-positive PNP sera (serum 3 to 12) specifically labelled transfected cells expressing recombinant A2ML1-c-myc with a fine granular cytoplasmic staining, while p170-negative PNP sera (serum 1 and 2) and normal sera (serum N1 and N2) did not (**Figure S2**).

Western blot analysis showed that HEK 293T cells transfected with the construct encoding A2ML1-c-myc expressed and secreted a protein of Mr 170,000 as expected (**Figure S3**). The secretion of the c-myc-tagged A2ML1 in the culture medium was considered as an indicator for the proper folding of the protein.

All p170-positive PNP sera (serum 3 to 12) immunoprecipitated A2ML1-c-myc from transfected HEK 293 cells, whereas p170-negative PNP sera (serum 1 and 2) did not (**Figure 4A**). The relative band intensities of the immunoprecipitates obtained from transfected cells using the different PNP sera paralleled those obtained using extracts from radiolabeled cultured keratinocytes. As control, sera from normal volunteers (n = 52), patients with

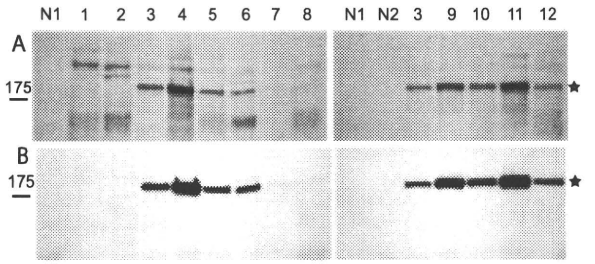

Figure 3. Anti-A2ML1 antibodies recognize p170 immunoprecipitated by PNP sera. Reduced samples immunoprecipitated from radiolabelled keratinocyte extracts with normal (N1, N2) or PNP sera (1 to 12) (see Fig. 1) were separated on 8% SDS-PAGE and transferred onto nitrocellulose membrane. This membrane was autoradiographed (A) and analyzed by immunoblotting using anti-A2ML1 antibodies (B).
doi:10.1371/journal.pone.0012250.g003

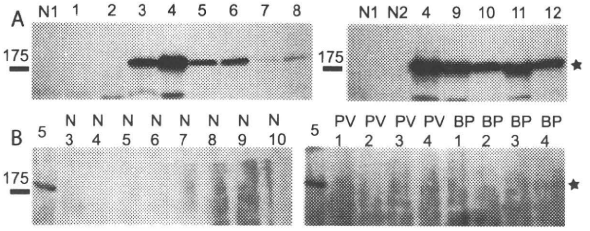

Figure 4. PNP sera immunoprecipitate recombinant A2ML1 expressed in HEK 293T. PNP (A, 1–12), normal (A and B, N1-N10), pemphigus vulgaris (PV1-PV4) and bullous pemphigoid sera (B, BP1-BP4) were used to immunoprecipitate proteins from extracts of HEK 293T cells transfected with pISb05, encoding recombinant A2ML1-c-myc. PNP sera numbering corresponds to that of Figure 1. Reduced immunoprecipitates were separated on 8% SDS-PAGE and analyzed by immunoblot using anti-c-myc antibody. p170 migration level is indicated by a star.
doi:10.1371/journal.pone.0012250.g004

pemphigus vulgaris (n = 24), pemphigus foliaceus (n = 4) and bullous pemphigoid (n = 28) did not immunoprecipitate A2ML1-c-myc from transfected HEK 293T cells (representative samples are presented **Figure 4B**). Together, these data provide strong support that p170 is A2ML1.

Adsorption studies demonstrate that p170 corresponds to A2ML1

To provide additional evidence that A2ML1 corresponds to p170, we carried out immunoprecipitation studies using PNP sera that were adsorbed with recombinant A2ML1- FLAG-His_8, expressed in transfected HEK 293T and bound to Ni^{2+}-resin. As control, serum samples were treated with a Ni^{2+}-resin loaded with mock-transfected cell extracts. To evaluate the selectivity of depletion, we first analyzed the pattern of immunoblotted proteins from undifferentiated and differentiated primary human keratinocyte extracts separated under non-reducing conditions (**Figure 5A**). Adsorbed PNP sera showed no or significantly reduced reactivity with p170, compared to mock-adsorbed PNP sera. Importantly, the binding of adsorbed sera to other PNP autoantigens was not affected, indicating that anti-p170 antibodies were selectively depleted by the A2ML1-FLAG-His_8-affinity resin. We then carried out immunoprecipitation studies using differentiated primary human keratinocytes (**Figure 5B**). The immunoprecipitates obtained either with mock-adsorbed or adsorbed PNP sera (n = 9) were probed by Western blotting using the anti-A2ML1 antibody. Immunoprecipitation of p170 was either completely or at least strongly reduced when adsorbed sera were used, compared to mock-adsorbed sera. Immunoblotting of the same immunoprecipitates with anti-envoplakin antibodies [17] confirmed that the adsorption was selective for anti-A2ML1 antibodies in PNP sera. These results indicate that PNP autoantibodies bind to common epitopes present on both p170 and A2ML1.

Finally, we also assessed the effect of the depletion of the anti-p170 antibodies by immunofluorescence microscopy of skin cryosections using the PNP serum no. 9 with strong and apparently selective reactivity with p170 (**Figure S4**). The mock-adsorbed PNP serum stained the upper and superficial epidermal cell layers, the pattern of which was similar to that obtained with the anti-A2ML1 antibody (**Figure S5**), as previously described [15]. In contrast, there was almost no labelling of the upper epidermal layers when the adsorbed PNP serum sample was used.

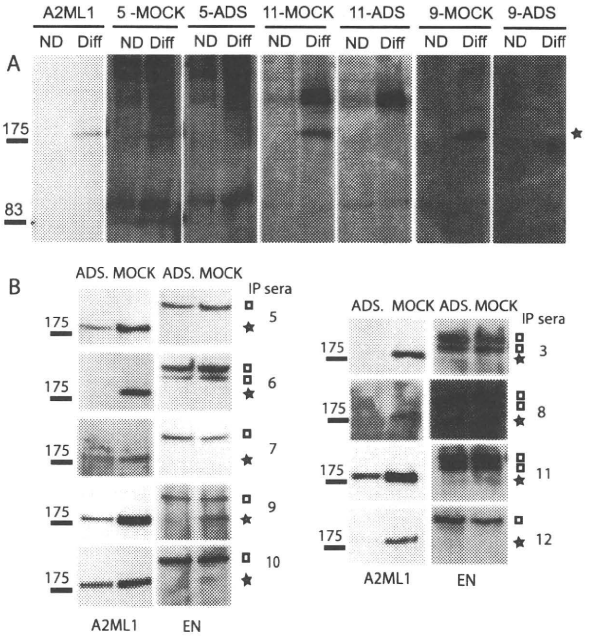

Figure 5. Adsorption of PNP sera with recombinant A2ML1 prevents PNP serum reactivity with p170. Nickel resin loaded with HEK 293T extracts expressing (ADS) or not (MOCK) A2ML1-Flag-HIS_8 was incubated with PNP sera (diluted 1/10). Sera numbering corresponds to that of Figure 1. (A) Treated sera were used to carry out the Western blot analysis of undifferentiated (ND) or differentiated for 8 days (Diff) human primary keratinocyte extracts (20 μg/lane), separated after non-reducing denaturation on 6% SDS-PAGE. The migration position of p170 is marked by a star. (B) Treated sera were used to immunoprecipitate proteins from 8-day-differentiated primary human keratinocytes. Reduced immunoprecipitates were separated on 6% SDS-PAGE, and analyzed by Western blotting using anti-A2ML1 antibody (A2ML1). The same membrane was reprobed with anti-envoplakin (EN). The one or two bands corresponding to envoplakin (17) are marked by a square. The sequential probing of the membrane without stripping explains the signal at p170 level when probed with the anti-envoplakin antibody.
doi:10.1371/journal.pone.0012250.g005

Mapping of A2ML1 domains recognized by PNP autoantibodies

To gain insight about potential mechanisms by which autoantibodies against A2ML1 may affect its function, we carried out a domain mapping study. A2ML1 was divided into two portions, based on the similarity between A2M and A2ML1 and A2ML1 predicted disulfide pattern [15], domains (http://smart.embl-heidelberg.de, email:) and secondary structures (GOR4 program developed by NPS@: network Protein Sequence Analysis) [18,19]. HEK 293T cells were transfected to express c-myc tagged recombinant forms of either the NH_2-terminal half (A2ML1 $^{1-889}$) or the COOH-terminal half of A2ML1 (A2ML1 $^{990-1454}$). The former encompasses the bait domain important for protease targeting, whereas the latter contains the thiolester and low density lipoprotein receptor-related protein 1-binding domains [15,20]. We assessed proper folding of these proteins by controlling their secretion (**Figure S3**). Nine out of ten PNP sera reacted with A2ML1 $^{1-889}$, while only three bound to the A2ML1 $^{990-1454}$, as revealed by immunoblotting of the immunoprecipitates with anti-myc antibodies (**Figure 6**). One serum did not show any reactivity. These results indicate that PNP autoantibodies mainly target the NH_2-half of A2ML1.

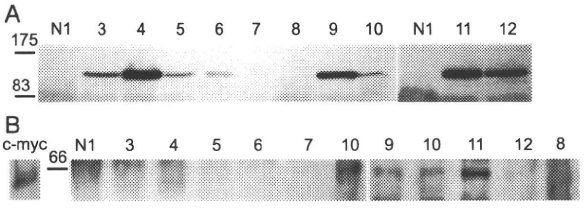

Figure 6. The NH_2-half of A2ML1 is preferentially recognized by PNP antibodies. PNP sera (1–12) and normal serum (N1) (see Fig. 1) were used to immunoprecipitate recombinant A2ML1$^{1-889}$-c-myc (A) or A2ML1$^{990-1454}$-c-myc (B) expressed in transfected HEK293T cells. Immunoprecipitates were separated by SDS-PAGE after reducing (A) or non-reducing (B) denaturation to avoid the co-migration of the protein of interest with the antibodies used for the immunoprecipitation and analyzed by Western blotting using anti-c-myc antibody. As a control A2ML1$^{990-1454}$-c-myc was immunoprecipitated in parallel with anti-c-myc antibodies (c-myc) and run together with the other samples. Exposure time of the c-myc lane was 2 sec versus 7 min for the others.
doi:10.1371/journal.pone.0012250.g006

Discussion

The characterization of p170 antigen recognized by PNP sera has proved to be a challenging task. Our study unravels the identity of this protein targeted by PNP autoantibodies as the protease inhibitor A2ML1. This conclusion is based on several lines of evidence: 1) the tryptic mass profile of the immunoprecipitated p170 has a significant match with that expected for A2ML1; 2) p170 is recognized by anti-A2ML1 antibodies and can be immunoprecipitated from culture media of human keratinocytes, in the same manner as A2ML1; 3) PNP sera immunoprecipitate recombinant A2ML1 from cell extracts, whereas binding to A2ML1 was never observed with sera obtained from normal volunteers (n = 52) as well as patients with autoimmune bullous diseases of the skin (n = 56); 4) p170-reactive PNP sera selectively labelled transfected cells expressing recombinant A2ML1; 5) preincubation of p170-reactive PNP sera with recombinant A2ML1 selectively abrogated reactivity of the PNP sera with p170 by immunoblot or immunoprecipitation, and further reduced the labelling of the epidermal granular cell layers, where A2ML1 is predominantly expressed. Together, our data unravel a novel class of proteins targeted by autoantibodies in patients suffering from this devastating multiorgan disease.

A2ML1 is a broad range protease inhibitor belonging to the A2M protease inhibitor family. It binds different classes of proteases and inhibits their activity or reduces the substrate spectrum by a "trap mechanism" in which the inhibitor covalently binds the protease and creates a sterical hindrance within the active site [15]. During this process, the conformational change releases the COOH-terminal extension of A2ML1, which then can be recognized by lipoprotein receptor-related protein 1 (LRP1) receptor to allow the internalization and clearance of the complex protease inhibitor-protease [20]. A2ML1 is expressed in many tissues such as the epidermis, thymus, and testis, while A2ML1 ESTs reported in UniGene mainly arise from normal and tumoral stratified epithelia [15]. Furthermore, the EST expression profile (Hs.620532, NCBI Unigene, EST profile viewer) suggests the presence of high transcript levels of A2ML1 in oesophagus, mouth, pharynx, intestine, and muscle. Interestingly, comparison of the sequence of A8K2U0 and CAD48670, which displays the highest score with p170 tryptic mass profile suggests the existence of splice variant(s) of A2ML1.

The exact function of A2ML1 is not yet defined. By analogy to A2M which has been proposed to be an element of the innate immunity [21] A2ML1 may participate in defense mechanisms by binding to inflammatory cytokines, growth factors and by targeting a broad range of proteases [15]. Furthermore, A2ML1 is likely to be directly implicated in the maintenance of epidermal homeostasis based on its ability to form covalent complexes with the kallikrein KLK7 *in vitro* [15], a protease involved in proteolysis of intercellular structures and in desquamation process [22].

Our findings provide an explanation for the failure to identify p170 so far. First, the full length cDNA sequence of A2ML1 in humans was reported in 2004 [23,24], while its functional characterization was carried out in 2006 [15]. Therefore, indexation and theoretical profiling of A2ML1 in databases have been only available in the past few years. Second, the biochemical properties of A2ML1 constitute an additional challenge for its identification. A2ML1 is a secreted glycoprotein with a secondary structure constrained by multiple disulfide bridges [15]. Furthermore, over-heating of A2ML1 in reducing-denaturing conditions results in its cleavage in two polypeptides of 120 kDa and 60 kDa ([15] and personal observations), which reduces the signal intensity at the expected electrophoretic migration. Finally, our analyses demonstrate that PNP anti-A2ML1 autoantibodies recognize conformational epitopes, explaining the two-decade failure to detect p170 by Western blot under denaturing conditions [7]. It is likely that production of A2ML1 in an eukaryotic expression system ensuring proper folding and posttranslational modifications facilitates its detection.

There is no ortholog of human A2ML1 in mouse, rendering the direct *in vivo* demonstration of the pathogenicity of anti-A2ML1 antibodies by passive transfer studies impossible. Accordingly, gene targeting experiments are not feasible. Furthermore, no hereditary human disorder has been mapped to the A2ML1 gene locus so far. Nevertheless, there are a number of indirect observations providing support to the idea that auto-antibodies against p170 are involved in the initiation or the perpetuation of tissue damage, since: 1) in a significant number of reported PNP patients, including patient 9 of the present study, autoantibody reactivity against p170 is found alone or with few additional reactivities, at least by immunoprecipitation [12,13]; 2) PNP sera binding to p170 can be detected already at an early stage of the disease [12,13]; 3) A2ML1 is not only expressed in skin but also in other organs affected in PNP (Hs.620532, NCBI Unigene, EST profile viewer); 4) acquired or genetic defects of protease inhibitors may cause a variety of muco-cutaneous diseases with systemic symptoms [22,25,26,27]. For example, mutations in SPINK5 gene encoding the serine protease inhibitor lympho-epithelial Kazal-type inhibitor (LEKTI) cause Netherton syndrome [26] associated with chronic skin inflammation and skin barrier defects. LEKT1 is thought to regulate kallikrein activity [28,29].

The impact of auto-antibodies on biochemical properties and function of A2ML1 could not be determined, due to difficulties to obtain sufficient amounts of purified A2ML1. However, two studies document the negative impact of the binding of autoantibodies to protease inhibitors. First, in acquired autoimmune angioedema, autoantibody binding to C1-inhibitor (C1-inh) facilitates its cleavage by its target proteases and result in a non-functional truncated circulating form of C1-inh [30]. Second, in rheumatoid arthritis, auto-antibodies to serpin E2 diminish the inhibitory activity of serpin on urokinase plasminogen activator serine protease [31]. In analogy, anti-A2ML1 antibodies may either destabilize A2ML1 or prevent its interaction with its target proteases, inflammatory cytokines or its receptor LRP1 and thereby affect the activity of extracellular proteases or amplify tissue damage. In this context, it should be noted that our domain mapping results show that the NH_2-terminal portion of A2ML1 is

almost systematically targeted by PNP autoantibody. Since this domain is implicated in the recognition of target proteases, PNP autoantibodies to A2ML1 may prevent the formation of protease-protease inhibitor complex. The identification of physiologically important targets of A2ML1 will be extremely useful to further understand the pathological involvement of anti-A2ML1 autoantibodies.

Dissecting the mechanisms underlying the association of PNP with distinct neoplasia is probably key for our understanding of the onset of autoimmunity in PNP. In the case of Castleman disease, tumor resection results in remission of PNP symptoms [2,32,33]. Castleman, thymoma or follicular dendritic cell sarcoma cells have been shown to produce autoantibodies reactive with various PNP autoantigens [33,34]. Noteworthy, reactivity with p170 is found in up to 76% of patients with PNP associated with Castleman disease [32,35]. Future systematic studies with prospective cohorts of patients and detailed analysis of the immunological profile are needed to assess whether presence of anti-A2ML1 autoantibodies is associated with a particular PNP phenotype and organ involvement as well as a specific type of neoplasia. In this context, the retrospective nature of our study precluded a reliable analysis. Nevertheless, based on the tissue distribution profile of A2ML1 and its lack of expression in pulmonary epithelium [36], it is unlikely that autoantibodies to A2ML1 contribute to bronchiolitis obliterans and respiratory failure, a frequent cause of death in PNP.

Our study thus puts an end to a relentless search for p170 and identifies PNP as a first example of an autoimmune multiorgan syndrom in which autoantibodies to a protease inhibitor might contribute to tissue damage by aggravating and precipitating inflammation.

Materials and Methods

Ethics Statement

Normal human serum samples were obtained from voluntary blood donors of the local regional Swiss blood bank. Written consent for public use was provided.

Patients' sera were obtained from patients managed in Switzerland (PNP n = 1, other bullous disease n = 56), France (PNP n = 12), Japan (PNP n = 7) and Germany (PNP n = 1). All sera were collected as a part of standard care and with the oral consent of the patients according to the local ethical rules. Sera were thus already available before this research was started. Since this is a non-interventional study and characterization of sera' reactivity is accepted and expected by the patients as normal diagnostic procedure, no written consent was required according to the local Swiss, French, German and Japanese ethics committees. Furthermore, since this research study did not involve intervention or interaction with the included individuals and the information collected during the study was not individually identifiable and not readily ascertained by the investigators, ethical review was not requested also in line with recent recommendations of the Office for Human Research Protections.

Human sera

Sera were obtained from 20 patients with clinically, histologically and immunopathologically typical PNP [2,3]. Sera were also obtained from patients with bullous pemphigoid (n = 28), pemphigus vulgaris (n = 24), pemphigus foliaceus (n = 4) and normal subjects (healthy blood donors, n = 52).

Cell culture and transfection

Primary human keratinocytes (Invitrogen) were grown in Keratinocyte-SFM medium, supplemented with EGF and pituitary extracts (Invitrogen), penicillin, streptomycine. Differentiation was induced by adding calcium 1 mM, isoproterenol 1 μM and hydrocortisone 0.4 μg/ml to the growth medium [37]. Human Embryonic Kidney cells HEK 293T (American Type Culture Collection) cells were grown in DMEM supplemented with foetal bovine serum (FBS) 10%, penicillin, and streptomycine. HEK 293T cells were transfected according to the calcium phosphate method [38]. When HEK 293T cell culture medium was analyzed for protein secretion, the cells were briefly rinsed in PBS 15 hours after transfection and grown in DMEM medium without FBS for two days.

In vivo labeling of keratinocytes

Primary human keratinocytes (foreskin) were purchased from Invitrogen. *In vivo* labelling of keratinocyte proteins was performed as previously described [10]. Briefly, after 4 days of differentiation, keratinocytes were incubated for 1 h in DMEM medium without methionin/cystein (Invitrogen) and then overnight in DMEM medium without methionin/cystein supplemented with 100 μCi/ml of ^{35}S-Met/^{35}S-Cys (Hartmann Analytics, Germany).

Cell extracts

Cells were lyzed in Tris-HCl 50 mM pH 7.5, NaCl 150 mM, NP-40 1%, protease inhibitor cocktail (Sigma). The insoluble fraction was removed by centrifugation, and the supernatant was used for either immunoprecipitation or, after denaturation in protein sample buffer with or without 2-mercapto-ethanol, for separation on SDS-PAGE (6%) and Western blotting.

Immunoprecipitation

Patient sera (30 μl) or anti-myc antibodies (4 μg) were incubated with protein A-Sepharose resin (GE Healthcare), in Tris-HCl 50 mM pH 7.5, NaCl 150 mM, protease inhibitor cocktail during 2 hours on ice. After discarding the unbound fraction, cell lysates were incubated with the resin 2 hours on ice, and then extensively washed with Tris-HCl 50 mM pH 7.5, NaCl 150 mM, protease inhibitor cocktail. Proteins bound to the resin were eluted in protein sample buffer (with or without 2-mercaptoethanol).

In-gel digestion of p170 for MALDI mass spectrometry analysis

The preparative 1D-SDS-PAGE gel, used to separate the immunoprecipitated proteins, was stained with Coomassie Brilliant Blue R250. The band containing the 170,000 protein was excised, and transferred into a fresh quartz vessel. The gel pieces were washed alternately three times with 10 μl digestion buffer (10 mM NH_4HCO_3, pH 7.8) and 10 μl modified digestion buffer (10 mM $NH4_HCO_3$/acetonitrile 1:1). Afterwards the gel pieces were shrunken in the vacuum and reswollen with 2 μl protease solution (0.05 μg/μl trypsin, Promega, USA). Digestion was performed for 10–12 h at 37°C. Afterwards 8 μl of 5% formic acid was added to the gel piece twice (successively) and the peptides were extracted for 15 min in a sonication bath. The pooled supernatant was mixed with 1 μl of C_{18}-beads (Poros 10 R2- chromatography beads, 5 mg/ml in methanol, PerSeptive Biosystems, USA) and completely dried in a vacuum concentrator. Concentrated samples were taken in 1 μl MALDI matrix (α-cyano-hydroxycinnamic acid in acetonitrile/0.1% TFA, 0.7:0.3).

MALDI mass spectrometry and data analysis

MALDI peptide mass fingerprint analysis was done on a Reflex IV mass spectrometer (Bruker Daltonics,Germany). Matrix containing C_{18}-bead bound peptides were transferred to a MALDI

sample plate. Dried samples were washed with 1 µl of 0.1% TFA. Analysis was done with the following settings: a target voltage of 20 kV, an acceleration electrode voltage of 13.5 kV, a reflectron voltage of 21.6 kV and a reflectron detector voltage of 1.6 kV.

PMF spectra were interpreted using the Mascot program (http://matrixscience.com) [16]. The chosen parameters were: human NCBI and MSDB database, tryptic cleavage, mass tolerance ±1.5 Da, expected mass of the protein 170 kDa. The number of maximal missed cleavages was set to 1.

Cloning

Clone DKFZp686O1010Q (AccNo AL832139) containing A2ML1 full length cDNA was purchased from ImaGenes (Germany). The vectors pISb05, pISb07, pISb08, pISb09 were derived from pcDNA3 vector (Invitrogen), and encode chimeric versions of A2ML1cDNA from clone DKFZp686O1010Q. pISb05 encodes A2ML1 fused to a c-myc-tag at its COOH-terminus, which was prepared in sequential steps. The c-myc-tag was introduced by PCR amplification using 5′GACTAGTCT-CACAGGTCCTCCTCTGAGATCAGCTTCTGTTCTGCTT-CACAGAGATCAGAATACTG and 5′CTTGCCAAATATGC-CACTAC primers. A strong Kozak sequence was also inserted by PCR using 5′CCCAAGCTTGCCGCCACCATGTGGGCT-CAGCTCCTTCTAG and 5′GAGAAGCCAAGGGAAACCTG primers. pISb07 is derived from pISb05, and encodes A2ML1 fused to a Flag-tag and a $His_{(8)}$-tag at its C-terminus: this double tag was introduced by PCR using 5′ TGGTGAGTCAGGGTC-TATGG and 5′TCCGGGCCCTCAATGATGATGATGAT-GATGATGATGACCGGTACGCTTGTCATCGTCATCCT-TGTAGTCTGCTGCTTCACAGAGATCAGAATACTG primers. pISb08 encodes A2ML1 (residues 1–889) ($A2ML1^{1-889}$) fused to a c-myc tag. This vector was derived from pISb05 and prepared using primers 5′ATGGGACAGACGTTTCTCTG and 5′ATAGGGCCCTCACAGGTCCTCCTCTGAGATCAGCT-TCTGTTCTGCTGCGGGAACAAACCCCTTCTG to insert the c-myc tag sequence. pISb09 encodes A2ML1 (residues 890–1454) ($A2ML1^{890-1454}$) fused to a C-myc tag, and was derived from pISb05. A strong Kozak sequence and a secretion signal peptide were introduced by PCR using 5′CCCAAGCTT-GCCGCCACCATGTGGGCTCAGCTCCTTCTAGGAATG-TTGGCCCTATCACCAGCCATTGCA CAAAAGGGCCGA-AGTGACAC and 5′GTAGAGGTTGGTCGTGGAGG primers. The sequence of the portions of cDNA prepared and amplified by PCR were verified by sequencing (Mycrosynth, Switzerland).

Preparation of protein from cell culture medium

Debris were removed from culture medium by centrifugation and proteins were precipitated with 10% v/v trichloroacetic acid 1 h on ice, the pellet was collected by centrifugation 10 min at 16,000 g, washed with acetone and resuspended in Laemmli sample buffer.

Protein reducing and non reducing denaturation and separation

Protein reducing denaturation was performed in Laemmli buffer [39] incubated 3 min at 95°C degrees. For the non reducing denaturation, 2-mercaptoethanol was omitted. SDS-PAGE separation was carried out as previously described [39].

Western blotting

Gels were transferred onto nitrocellulose membrane in Tris-glycine buffer, ethanol 10%, 0.1%SDS, for 1 h at 100 V. Western blot analyses were performed in standard conditions. Antibodies were diluted as follows: anti-A2ML1 1/250 (Abnova), anti-Flag 1/500 (Sigma-Aldrich), anti-c-myc 1/500 (Santa Cruz Biotechnology).

Serum adsorption

Soluble extracts from HEK 293T cells transfected with A2ML1-FLAG-His_8 were prepared as described above using a pH 8 buffer and were incubated with Nickel-resin (Sigma-Aldricht) 1 h on ice, and the resin was subsequently submitted to extensive washing with Tris-HCl 50 mM, pH 8, NaCl 200 mM, NP40 1%, protease inhibitor. The equilibrated resin was incubated overnight with 100 µl serum diluted 10 fold in the same buffer with protease inhibitors. The same procedure was performed using untransfected HEK 293T cell extracts for the control experiment. The supernatant was then used for Western blot analysis, immunoprecipitation or immuno-fluorescence.

Immunofluorescence

All antibodies were diluted in PBS 1% BSA. HEK 293T cells were fixed with paraformaldehyde 4%, permeabilized with Triton X-100 0.1%, blocked with BSA 1%, and incubated with Anti-c-myc (1/150) and patient sera (1/150) during 1 h. The Alexa-fluor 488-anti-rabbit and Alexa-fluor-588-anti-human secondary antibody (Invitrogen) were diluted 1/300 and incubated 1 h at room temperature. Nuclei were stained with DAPI. Cryostat sections (4–5 µm) of human breast skin were air-dried, fixed for 10 min in 4% ice cold acetone and rehydrated in Tris buffered saline with 0.1% saponin. Double immunofluorescence was performed by serially incubating sections with anti-A2ML1 (1/50) and anti-envoplakin CR5 (1/100) antibody for 1 h followed by incubation with Alexa-fluor-568-anti-mouse (1/100) and Alexa-fluor-588-anti-rabbit (1/100). Nuclei were stained with DAPI. Immunofluorescence with adsorbed sera was performed incubating the adsorbed and mock-adsorbed sera (1/2.5) 1 h, and then Alexa-fluor-568 anti-human antibody (Invitrogen).

Supporting Information

Figure S1 Monoisotopic mass profiles of p170 tryptic digest (MALDI-TOF mass spectrometry).
Found at: doi:10.1371/journal.pone.0012250.s001 (9.64 MB EPS)

Figure S2 PNP sera selectively recognize HEK 293T cells expressing recombinant A2ML1-c-myc. Cells transfected with pISb05, encoding A2ML1-c-myc were labelled with both anti-c-myc antibody (green) and human sera (red): normal (A) or PNP 2, 4 and 7 (B, C and D). Nuclei were stained with DAPI (blue). Sera numbering corresponds to that of Figure 1. White scale bar: 100 µm.
Found at: doi:10.1371/journal.pone.0012250.s002 (2.14 MB EPS)

Figure S3 Expression and secretion of recombinant A2ML1 proteins in HEK 293T cells. Extracts (20 µg) of untransfected HEK 293T cells (lanes 1) or transfected with pISb07 (lanes 2), encoding A2ML1-Flag-His8, were separated on 10% SDS-PAGE, and analyzed by Western blotting using anti-A2ML1 or anti-Flag antibody. B. Culture medium (400 µl) of 293T cells non transfected (lane 1), or transfected with pISb05 (lane 2), pISb08 (lane 3), pISb09 (lane 4) encoding A2ML1-c-myc, A2ML11-889-c-myc, and A2ML1890-1454-c-myc, respectively were TCA-precipitated, separated on 10% SDS-PAGE and analyzed by Western blotting using anti-c-myc antibody. Lane 2, the band at 60,000 is likely a COOH-terminal degradation product of A2ML1 occurring during denaturation of the sample under reducing conditions.
Found at: doi:10.1371/journal.pone.0012250.s003 (1.95 MB EPS)

Figure S4 Adsorption of PNP-serum 9 with recombinant A2ML1 reduces immunofluorescence staining in the upper layers of epidermis. PNP serum 9 mock-adsorbed (A) or adsorbed with recombinant A2ML1 (B) were used to probe sections of human breast skin. The reactivity of the secondary anti-human antibodies against human breast skin section is shown (C): the signal detected in the dermis and the upper cornified edge of the epidermis is therefore non-specific to the patient serum reactivity. The specific reactivity due to A2ML1 is expected in the upper layers of the epidermis, areas indicated by the white arrows. As a reference A2ML1 pattern in human breast skin is shown. Nuclei were stained with DAPI (blue). White scale bar: 100 μm.
Found at: doi:10.1371/journal.pone.0012250.s004 (2.19 MB EPS)

Figure S5 A2ML1 is expressed in the upper layers of human skin. Sections of human breast skin were labeled with CR5 anti-envoplakin antibody (A, green) and anti-A2ML1 antibody (B, red). The patterns obtained with the secondary anti-rabbit (C, green) and anti-mouse antibody (D, red) are shown. Nuclei were stained with DAPI. White scale bar: 100 μm.

Found at: doi:10.1371/journal.pone.0012250.s005 (1.74 MB EPS)

Acknowledgments

We are very grateful to Dr. D. Gilbert and Prof.P. Joly (Tours, France), Prof. M. Amagai, Dr. K. Ishii (Tokyo, Japan), and Prof. M. Hertl (Marburg, Gemany) for providing PNP serum samples. Prof. T. Hunziker and Dr. S. Fontana (Berne, Switzerland) kindly provided serum samples from patients with autoimmune bullous skin diseases and normal subjects. Prof. H.E. Meyer (Frankfurt, Germany)is kindly thanked for the mass spectrometry service provided. We are also thankful to Dr. L. Fontao (Geneva, Switzerland) for helpful discussions. The polyclonal antiserum CR5 against envoplakin was a gift of Prof. F. Watt (London, UK). We are grateful to Prof. K. Yancey (Dallas, USA) for the critical reading of the manuscript.

Author Contributions

Conceived and designed the experiments: IS BF LB. Performed the experiments: IS FJ NB UL KM. Analyzed the data: IS. Contributed reagents/materials/analysis tools: KM TH. Wrote the paper: IS LB.

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