Pemphigus

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Laboratory diagnosis of paraneoplastic pemphigus

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Abstract

Background Paraneoplastic pemphigus (PNP) is a multiorgan disease characterized by antibodies against plakins, desmogleins and the alpha-2-macroglobulin-like-1 (A2ML1) protein, in association with an underlying neoplasm. Accurate diagnosis relies on the demonstration of these autoantibodies in serum.

Objectives To evaluate the value of different laboratory techniques in the serological diagnosis of PNP.

Methods We performed immunoblotting, envoplakin (EP)-ELISA, indirect immunofluorescence (IIF) on rat bladder, radioactive immunoprecipitation and a non-radioactive combined immunoprecipitation-immunoblot assay. Additional assays included BP180-ELISA and BP230-ELISA. We included sera of 19 PNP and 40 control patients.

Results The sensitivities were 63% for anti-EP ELISA, 74% for rat bladder IIF, 89% for immunoblotting, 95% for radioactive immunoprecipitation and 100% for non-radioactive immunoprecipitation. Specificities ranged from 86-100%. The BP180- and BP230-ELISAs had low sensitivity and specificity for PNP. The combination of rat bladder-IIF and immunoblot showed 100% sensitivity and specificity. Analysis of sequential PNP sera showed that antibody titers may decrease over time, possibly resulting in negative outcomes for envoplakin-ELISA and rat bladder-IIF studies.

Conclusions Detection of autoantibodies against envoplakin and periplakin, or A2ML1 by immunoprecipitation is most sensitive for PNP. The combination of rat bladder-IIF and immunoblotting is equally sensitive and highly specific, and represents an alternative valuable and relatively easy approach for the serological diagnosis of PNP.
3. Laboratory diagnosis of paraneoplastic pemphigus

**Introduction**

Paraneoplastic pemphigus (PNP) is a severe autoimmune multiorgan disease, first described by Anhalt et al in 1990.  
It is characterized clinically by painful stomatitis, polymorphous cutaneous manifestations and sometimes also pulmonary involvement, in patients with underlying neoplasia.

Mortality rates are high, with 1-, 2-, and 5-year survival rates of 49%, 41% and 38% respectively, the main causes of death being infections and progression of neoplasia.

Histological changes are numerous, including intraepidermal acantholysis, vacuolar interface dermatitis and keratinocyte necrosis.

Direct immunofluorescence (DIF) studies of skin or mucosa biopsies often show deposition of IgG and complement component C3 on the epithelial cell surface, sometimes accompanied by linear depositions along the epithelial basement membrane zone (BMZ).

The manifestations of PNP are therefore diverse, resembling pemphigus vulgaris (PV), erythema multiforme, Stevens-Johnson syndrome, toxic epidermal necrolysis or a lichenoid dermatosis.

This is why diagnosis is often challenging.

PNP is also characterized by the presence of autoantibodies against multiple antigens, among which are the proteins of the plakin family, which are part of the intracellular plaque of desmosomes and/or hemidesmosomes. These include the 210 kDa envoplakin (EP), the 190 kDa periplakin (PP), the 230 kDa bullous pemphigoid antigen BP230, 250 and 210 kDa desmoplakin I and II (DSPI and DSPII), and the 500 kDa plectin. The previously described 170 kDa autoantigen has recently been identified as the protease inhibitor alpha-2-macroglobulin-like-1 (A2ML1). Other antigens include the desmogleins (Dsg) 1 and 3. Antibodies to plakophilin 3 and desmocollins 1-3 have also been reported. Demonstration of PNP specific antibodies is an important tool in diagnosing PNP. Several laboratory tests are available, including indirect immunofluorescence microscopy (IIF) studies on rat bladder sections. Rat bladder urothelium does not contain Dsg1 and -3 but does contain DSP, EP and PP. A positive staining of the urothelium is therefore an indication of PNP. Immunoprecipitation and immunoblot are often used to demonstrate the characteristic PNP antibody response. A relatively new test is anti-EP ELISA that has recently become commercially available. Tests to characterize the anti-BMZ antibody response include a BP230- and BP180-ELISA. Although these techniques have been individually validated, comparative studies on their usefulness in diagnosing PNP are scarce. As PNP is rare, with only approximately 450 cases reported so far, and as it only comprises 3-5% of all pemphigus cases (personal estimation based on Dutch patients), most laboratories are not familiar with its diagnostics. In the present study we therefore compared the diagnostic value of the currently available immunoserological techniques.
Materials and Methods
Patient selection
Nineteen PNP patients were included, with a median age of 56.6 years (age range 29-86 years). The male to female ratio was 2:1. The diagnosis of PNP was made if patients fulfilled the following criteria, adapted from the revised criteria proposed by Anhalt in 2004 and Zimmerman in 2010: (1) Painful and persistent stomatitis, with or without a polymorphous skin eruption. (2) The demonstration of plakin autoantibodies. (3) The presence of an underlying neoplasm. As control, twenty-four PV patients were included, one of whom had an underlying neoplasm (lung carcinoma) but no other PNP characteristics. Furthermore, three patients who had additional anti-DSP antibodies (a-DSP) (one pemphigus foliaceus, one PV and one bullous pemphigoid), and 13 toxic epidermal necrolysis (TEN) patients were included as controls. The diagnosis of PV was based on the clinical and histological features of suprabasal acantholysis and the presence of anti-Dsg3 with or without anti-Dsg1 antibodies. The diagnosis of TEN was based on clinical and histological features. If DIF biopsies of PNP and PV were available these showed the typical pemphigus epidermal anti-cell surface (ACS) deposition pattern of IgG with or without C3 deposits. Most PNP patients had additional depositions of IgG or C3 along the BMZ. PNP patient characteristics are summarized in table 1.

Immunoblot, radioactive and non-radioactive immunoprecipitation analyses
Routine immunoblot analysis was performed as described previously. Radioactive immunoprecipitation analysis was performed using radiolabeled extracts from cultured differentiated human keratinocytes, as previously described. For the non-radioactive immunoprecipitation analysis, keratinocytes grown to near confluence, were differentiated for 6 to 10 days to induce A2ML1 by adding 1 mM CaCl2, 0.1 mM isoproterenol and 0.4 μg/ml hydrocortisone to CnT keratinocyte medium (Celltech, Bern, Switzerland). Cells were then extracted for 10 minutes in ice cold 1% (v/v) Triton-X100, 50 mM MOPS, 150 mM NaCl, 5 mM EDTA with protease inhibitor Complete (Roche, Almere, the Netherlands). Debris was removed by centrifugation and the extract was stored at -80 °C. Patient serum (20 μl) was incubated for one hour at room temperature with 60 μl GammaBind G Sepharose (GE healthcare, Uppsala, Sweden) and washed over a 1 M sucrose plus 150 mM NaCl solution. One hundred μl keratinocyte extract was incubated with the beads for one hour at room temperature, and washed over sucrose solution. Twenty μl Laemmli sample buffer was added to the beads and heated at 100 °C for 5 minutes. This was run on a 5% SDS-PAGE slab gel and then electrophoretically transferred to an Immobilon-P PVDF-membrane (Millipore, Billerica, MA, U.S.A.). The membrane was blocked with 2% (w/v) low-fat milk powder TBST for 1 hour and incubated overnight with a cocktail of 1:500 diluted antibodies against DSP (DPSII.15; Abcam, Cambridge, U.K.), EP (CRENV-1; Abcam), PP (C20; Santa Cruz Biochemicals, CA, U.S.A) and a 1:250 diluted anti-A2ML1 antibody (B01P, Abnova, Taipei City, Taiwan). Subsequent incubations consisted of 1:2000 goat anti-rabbit IgG (Nordic Immunologic Laboratories; Eindhoven, the Netherlands) and 1:500 AffiniPure goat anti-mouse IgG (Jackson Immunoresearch, West Grove, U.S.A), followed by 1:500 alkaline phosphatase conjugated AffiniPure rabbit anti-goat IgG (Jackson Immunoresearch, West Grove, U.S.A.). Antibody binding was visualized by incubation with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.
ELISA

ELISAs for the detection of anti-Dsg1, -Dsg3, the NC16A ectodomain of BP180, BP230 (all MBL, Nagoya, Japan) and the N-terminal domain of EP (EUROIMMUN, Lubeck, Germany) IgG autoantibodies were performed according to the manufacturer’s instructions.

Indirect immunofluorescence microscopy

Indirect immunofluorescence studies of patient sera was performed on rat bladder (1:10) as previously described. As secondary antibody we used FITC-conjugated goat anti-human IgG (Protos Immunoresearch, Burlingame, CA, U.S.A.). Sections were viewed under a Leica DMRA fluorescence microscope and images were recorded with a Leica DFC350 FX digital camera (Leica, Wetzlar, Germany).

Statistical analysis

The sensitivities and specificities of the different immunoserological techniques to diagnose PNP were calculated as follows:

Sensitivity (%) = True positive/(True positive + False negative) x 100
Specificity (%) = True negative/(True negative + False positive) x 100

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<th>Patient #</th>
<th>Age (yrs.)/sex</th>
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<th>Dsg3 ELISA index</th>
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<td>&gt;150</td>
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<td>94</td>
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</table>

Table 1. PNP patient characteristics. Abbreviations: CLL, Chronic Lymphocytic Leukemia; NHL, Non-Hodgkin Lymphoma; yrs., years; ELISA, enzyme linked immunosorbent assay; DIF, direct immunofluorescence microscopy; ACS, anti-cell surface; BMZ, basement membrane zone; G+, positive for IgG depositions; C+, complement depositions, M+, IgM depositions, A+, IgA depositions; n.a., biopsies not available; -, negative.
Results

Immunoprecipitation using radioactively labeled keratinocyte extracts
When tested by radioactive immunoprecipitation, 14 PNP sera (74%) precipitated EP and PP and 15 PNP sera (79%) precipitated A2ML1 (Fig. 1a). Overall, 18 PNP sera (95%), but none of 8 control sera, precipitated either EP and PP or A2ML1. Thirteen (68%) PNP sera precipitated DSP and 4 patients (21%) BP230. One serum (#15) only precipitated DSP, with two bands of 250kDa and 210kDa representing DSP-I and –II, respectively, and could not be distinguished from the three a-DSP controls that also precipitated DSP. This serum however was positive on immunoblot (IB) and by non-reactive immunoprecipitation-immunoblot (IP-IB) for antibodies against EP and PP.

Non-radioactive immunoprecipitation-immunoblot analysis
To evaluate a non-radioactive combined IP-IB technique for PNP, we tested sera of 15 PNP (Fig. 1b), 12 PV, 13 TEN, and the 3 a-DSP control patients. Four PNP sera (#1, 2, 3 and 13) could not be tested due to the limited amount of serum. Thirteen PNP sera (87%) precipitated EP and PP, 7 (47%) of these 13 also precipitated A2ML1, and three (20%) precipitated DSP. Two sera (#18 and 19) tested positive for A2ML1 by nr-IP and IP-IB, but tested negative for EP and PP by all techniques. Overall, all 15 PNP sera tested positive for either anti-EP and –PP antibodies or anti-A2ML1 antibodies. None of the 28 controls precipitated EP, but two PV sera precipitated PP. Surprisingly, with four of the 13 TEN sera we observed a faint band at the position of A2ML1. The intensities of these bands were considerably weaker than those obtained with the PNP sera. Two of these four also weakly precipitated PP. DSP was precipitated by one PV and by one of the three a-DSP sera.

Immunoblotting
By IB, using protein extracts from undifferentiated keratinocytes, 17 of the 19 (89%) PNP sera bound to EP and PP (Fig. 1c). Binding to DSP-I and –II was seen for respectively 9 (47%) and 7 (37%) cases. Six sera (32%) bound to BP230 and one (5%) bound to plectin. In the control group, one of 24 PV sera recognized BP230 (4%), while all 3 a-DSP sera bound DSP. The 12 included TEN sera all tested negative. An overview of the r-IP, IP-IB and IB results for the PNP and control group is shown in Fig.1d. The presence of EP and PP, or A2ML1 autoantibodies was valued as positive outcome and used in the calculation of the sensitivity and specificity of each test (Table 3).

EP ELISA
Twelve PNP sera (63%) tested positive by EP-ELISA (Fig. 2a). One of the 24 PV controls also tested positive, but no evidence for EP antibodies was found by either IB or IP-IB and rat bladder IIF was negative. All TEN sera tested negative. Of 5 PNP patients consecutive sera were available. EP-ELISA of these sera showed that anti-EP titers decreased during immunosuppressive therapy, and for two patients (# 6 and 7) the EP-ELISA index dropped below the cut-off of the ELISA (Fig. 2b). Nevertheless EP antibodies could still be demonstrated by IB, r-IP and IP-IB.
Indirect immunofluorescence on rat bladder
Rat bladder IIF was considered positive if sera bound to the urothelial cell surface (Fig. 3a) with or without binding to the basement membrane zone. Some PNP sera bound in a smudgy urothelial pattern, but, as this was also seen for some controls, we considered this a negative test. Two of three a-DSP sera gave subtle urothelial cell surface staining of luminal cells (Fig. 3b), which was different from the PNP pattern and also valued as negative. Fourteen of the 19 PNP patients (74%) tested positive and all PV, TEN, and a-DSP sera tested negative. The two sera (#18 and 19) that tested negative for PNP-specific antibodies by IB and EP ELISA, but positive for anti-A2ML1 antibodies by r-IP and IP-IB, tested positive on rat bladder. For one patient (#6) from whom we had consecutive sera, the test became negative during treatment.

Additional assays for BMZ antigens
Direct IF studies of PNP patients’ skin specimens sometimes demonstrate the presence of BMZ IgG deposition in addition to epidermal ACS deposition. Therefore, we tested all PNP and PV sera by BP230 and NC16A ELISA. Two PNP and three PV sera tested positive in the B230-ELISA, and 5 PNP sera and one PV serum tested positive in the NC16A-ELISA. No clear correlation between DIF IgG BMZ depositions and ELISA was observed.
All data are summarized in tables 2 and 3. Based on these data we formulated a diagnostic algorithm for PNP, as illustrated in figure 4.
Figure 1 Autoantibodies to plakins and anti-A2ML1 detected by r-IP, IP-IB and IB.

(a) Radioactive immunoprecipitation (r-IP) results for 19 PNP sera, (b) Non-radioactive immunoprecipitation-immunoblot (IP-IB) results for 15 PNP sera. For sera # 18 the A2ML1 band was weakly visible by eye, but barely visible after scanning to a digital image. We therefore used software to further enhance this image. (c) Immunoblot (IB) results for 19 PNP sera. (d) The prevalence of the different anti-plakin and anti-A2ML1 antibodies in the PNP and control group, as measured by r-IP, IP-IB and IB.
3. Laboratory diagnosis of paraneoplastic pemphigus

Figure 2 Envoplakin autoantibody titers as measured by ELISA.
(a) Twelve PNP and 1 control patient had titers above the ELISA detection limit. (b) Titers decrease over time. Two of five tested PNP patients had titers decreasing below the ELISA detection limit.

Figure 3. Indirect immunofluorescence on rat bladder urothelium.
(a) Positive urothelial cell surface staining pattern typical for PNP. (b) Anti-DSP control serum gives a subtle luminal staining pattern (white arrows) of rat bladder urothelium. All scale bars represent 20 micrometers.
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Table 2. Overview of test outcomes for PNP.
Abbreviations: Pat. #, patient number; Rb-IIF, rat bladder indirect immunofluorescence; ELISA, enzyme-linked immunosorbent assay, r-IP, radioactive immunoprecipitation; IP-IB, immunoprecipitation-immunoblot assay; IB, immunoblot; DSP, desmoplakin; EP, envoplakin; PP, periplakin; A2ML1, alpha-2-macroglobulin-like-1; Nd, test not done; -, negative test outcome; +, positive test outcome.

Table 3. Sensitivity and specificity of different laboratory techniques for PNP
Abbreviations: r-IP, radioactive immunoprecipitation; IP-IB, immunoprecipitation-immunoblot; IB, immunoblot; IIF, indirect immunofluorescence; ELISA, enzyme-linked immunosorbent assay; PV, pemphigus vulgaris; a-DPK, control patients with anti-desmoplakin antibodies; TEN, toxic epidermal necrolysis; EP, envoplakin; PP, periplakin; A2ML1, alpha-2-macroglobulin-like-1.
Figure 4. Diagnostic algorithm for PNP.
Abbreviations: IIF-rat bladder, indirect immunofluorescence on rat bladder; TEN, toxic epidermal necrolysis; EP, envoplakin; PP, periplakin; A2ML1, alpha-2-macroglobulin like-1. A positive IIF-rat bladder is defined as positive urothelial cell surface staining. A positive immunoblot outcome is defined as serum reactivity to both envoplakin and periplakin.
Discussion

In this study we compared different assays for the serological diagnosis of PNP. Historically, the IP assay using radioactively labeled keratinocyte extracts was the first technique used to identify PNP autoantibodies. We show that the sensitivity of this technique is superior to IB, IIF on rat bladder, and EP-ELISA. However, because of its radioactivity, this technique is not widely available. We therefore developed a non-radioactive IP-IB assay. Although the comparison is limited by the smaller number of PNP patients we used for the non-radioactive IP assay, our results suggest that this assay is slightly more sensitive than the radioactive IP and similarly specific. Another advantage of our non-radioactive IP assay is that we confirm the identity of the autoantigens not only based on size but by using specific anti-plakin and A2ML1 antibodies to visualize the blotted precipitates. Therefore, this assay is an addition to the other non-radioactive IP assays that have been developed in previous PNP studies. The sensitivity of IB analysis closely follows that of r-IP and IP-IB studies. By r-IP we missed one and by IB two of our 19 PNP patients. The patients that were missed by IB had antibodies to A2ML1, in addition to other non-PNP-specific antibodies. The reason that these cases were missed is that our routine diagnostic blots are prepared with low-calcium grown cells and reduced substrate, while A2ML1 is produced by cells that are grown for several days at high calcium and is only recognized by autoantibodies under non-reducing conditions. The serum that was false-negative in the r-IP possibly had too low anti-EP and anti-PP titers. The EP ELISA had a lower sensitivity and specificity when compared to IP and IB studies. Our results from sequential PNP sera further show that anti-envoplakin antibody titers may decrease over time, sometimes resulting in a negative ELISA as titers drop below the cut-off. This decrease may be due to immunosuppressive therapies, or may be part of the natural disease course. Therefore, the possibility of seroreversion should be considered when interpreting envoplakin-ELISA results from suspected patients from whom serum is taken later in the course of disease. Serum IF on rat bladder substrate is an accurate test to differentiate PNP from PV, as rat bladder epithelium does not contain Dsgs, but does express plakins. In the present study rat bladder IIF was more sensitive than EP-ELISA. Notably, two patients that tested negative for anti-EP and anti-PP antibodies by IB and ELISA, tested positive by rat bladder IIF. The sera of these patients contained anti-DSP and anti-A2ML1 antibodies as shown by radioactive IP. Rat bladder does not contain an A2ML1 orthologue, and anti-DSP antibodies give a different staining pattern than that seen for these two sera. Therefore, this positive urothelial staining suggests that more -still unknown- PNP antigens are present in rat bladder. In our group the combination of IB and rat bladder IIF led to 100% sensitivity and specificity. Both techniques are faster, less labour intensive, and require less patient’s serum than IP assays. However, rare PNP cases with only anti-A2ML1 antibodies might be missed by the combination of IB and rat bladder IIF. Therefore, for patients clinically suspect for PNP but with negative IB and rat bladder IIF, IP might be useful. The detection of solitary anti-A2ML1 antibodies by IP may not always discriminate between PNP and TEN because TEN sera may also produce very weak bands at the position of A2ML1. However, our findings do suggest that anti-A2ML1 antibody titers are much lower in TEN than in PNP. Studies aimed at developing an A2ML1-ELISA might therefore prove fruitful to further quantify these differences. Similar to the EP-ELISA, use of medication led to a negative rat bladder test for one of the patients, indicating
that concurrent use of medication might also reduce the sensitivity of rat bladder-IIF. Our results show that the antibody response in PNP is variable, since it can be directed to the complete spectrum of PNP antigens but can also be limited to part of it. We confirm that the combined presence of anti-EP and anti-PP antibodies is most specific and sensitive for PNP, and that solitary anti-A2ML1, anti-PP, anti-DSP and anti-BMZ antibodies are not. We therefore propose that the diagnosis PNP should not be solely based on clinical, histological, DIF or unspecific IIF findings, as suggested by various studies, but that the demonstration of antibodies to EP and PP, or a positive rat bladder IIF is necessary.

In conclusion, our findings indicate that IP studies are most sensitive in detecting PNP specific autoantibodies. Rat bladder IIF, although less sensitive, is a relatively simple technique, which may be of complementary value. In settings where IP studies are not available, the combination of IB and rat bladder-IIF should be used as first serological analyses for confirming the diagnosis of PNP. Finally, when evaluating laboratory results, one should consider that, possibly due to therapy or natural disease course, antibody titers may decrease over time. Further studies should determine if specific antibody profiles or IIF staining patterns are related to specific PNP phenotypes and might predict disease outcome. This would provide more insight in PNP pathogenesis and treatment.
References
