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Comparative study of five serological assays for the diagnosis of paraneoplastic pemphigus

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Summary

Paraneoplastic pemphigus (PNP) is an autoimmune mucocutaneous blistering disease driven by autoantibodies against plakins expressed in mucosal epithelium. Diagnosis can be difficult as both clinical and biopsy features overlap with other blistering disorders, thus serology is important. Indirect immunofluorescence (IIF) on rat bladder substrate is the most widely used assay, but plakin-specific autoantibody assays have recently become available. The aim of this study was to compare the performance of five PNP assays in patients with mucosal blistering disease: IIF with rat bladder, monkey bladder and rat cardiac substrates, an envoplakin enzyme-linked immunosorbent assay (ELISA), and an envoplakin-transfected HEK cell based assay (CBA). Fifty-one patient serum samples, comprising three PNP patients and 48 disease controls, were collected along with 10 healthy control samples, and analysed using the five assays. IIF on rat and monkey bladder substrates both showed high specificity (97% and 95%, respectively), and correctly identified all three PNP sera. The envoplakin ELISA was equally specific (98%) but identified only one PNP patient. The CBA was difficult to interpret, and both this assay and IIF on rat cardiac substrate lacked specificity (82% and 83%, respectively). In this study IIF using either rat or monkey bladder substrates performed strongly, whilst the envoplakin ELISA seemed to lack sensitivity, and the CBA and IIF on rat cardiac substrate were inferior. Our findings suggest that traditional IIF-based assays remain the preferred approach in the serological diagnosis of PNP.

Key words: Envoplakin, monkey bladder, oral ulceration, paraneoplastic pemphigus, pemphigus, periplakin, rat bladder.

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INTRODUCTION

Paraneoplastic pemphigus (PNP) is a rare autoimmune blistering condition involving the skin and mucous membranes. It has a universal association with malignancy and a poor prognosis.[1] It was first described in 1990[2] and since then over 450 cases have been reported.[3] The majority of patients present following the diagnosis of malignancy, but occasionally the malignancy may initially be occult.[4] In the latter case, the diagnosis of PNP can be particularly challenging, as both the clinical presentation and biopsy findings overlap with other conditions associated with mucocutaneous ulceration;[4–7] in such circumstances, serology plays an important diagnostic role.

PNP is driven by the action of multiple autoantibodies, including those seen in other immune-mediated blistering conditions, such as pemphigus vulgaris (desmoglein-1 and -3) and pemphigoid (bullous pemphigoid antigen-230); however, anti-plakin autoantibodies seem to be specific for this condition.[2] The plakin family constitutes a number of homologous proteins located in intermediate filaments and filament attachment sites at the plasma membrane, and includes periplakin (190 kDa), envoplakin (210 kDa), desmoplakin-1 (250 kDa),[2] and the recently described 170 kDa protease inhibitor alpha-2-macroglobulin-like-1 (A2ML1).[8] Autoantibodies to all family members have been detected and characterised in PNP using radioimmunoprecipitation (RIP).

A number of alternative assay methodologies, arguably more suited to the routine diagnostic laboratory, are also available. Helou *et al.*[9] evaluated various murine tissue substrates using IIF and found that positive staining of the plakin-rich transitional epithelial cells on rat bladder was a reasonable alternative to RIP. However, given its inferior sensitivity (75%), the authors recommended retaining RIP for cases with strong clinical suspicion. Since other substrates such as rat heart and rat liver performed less well,[9] IIF on rat or monkey bladder substrate has been the accepted assay for the routine diagnostic laboratory. Recently however, plakin-specific assays have appeared, particularly anti-envoplakin, with the theoretical advantage of being quantitative and automatable, yet there is a paucity of head-to-head comparative studies in heterogeneous populations with miscellaneous blistering disease.

MATERIAL AND METHODS

This audit was approved by the Western Sydney Local Health District Ethics Committee, Westmead Hospital, Westmead.

Patient selection

The Oral Immunology Clinic at Westmead Hospital in Sydney, Australia, is a quaternary referral centre for patients with recalcitrant oral ulceration, and is attended by clinical immunology and oral medicine specialists. Fifty-one patients were included in the present study, three with PNP and 48 disease controls. The latter group comprised 18 patients with pemphigus vulgaris (PV), 12 with mucous membrane pemphigoid (MMP), eight with oral lichen planus (OLP), three with Behçet's disease, three with recurrent apthae, one with recurrent erythema multiforme and one patient with chronic gingivitis.

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Diagnosis was based on accepted criteria, including oral mucosal biopsy where appropriate. Ten serum samples from healthy controls were also included.

Of the three PNP patients, two had a history of B-cell lymphoma. The third, who was HIV negative, was diagnosed with unicentric Castleman's disease following biopsy of a pelvic mass. All three suffered from refractory oral ulceration and demonstrated histological findings compatible with a diagnosis of paraneoplastic pemphigus. Only two of 48 disease controls, both of whom had MMP, also had a history of malignancy (transitional cell carcinoma of the bladder and B-cell lymphoma), both of which were in remission at the time of the study. One other MMP patient had a low level paraprotein (1–2 g/L) and another had an incidental finding of anti-Yo antineuronal antibody, but without the associated clinical manifestations. Many patients were on immunosuppression at time of testing but all were deemed to have some degree of disease activity by their treating physicians.

Assays

Four assays including indirect immunofluorescence (IIF) on rat bladder substrate (Euroimmun, Germany), IIF on monkey bladder substrate (Inova, USA), IIF on rat cardiac substrate (Euroimmun) and envoplakin enzyme-linked immunosorbent assay (ELISA; Euroimmun) were performed on all 61 samples. The IIF cell-based assay (CBA), using envoplakin-transfected HEK293 cells as substrate (Euroimmun), was performed only on the first 28 serum samples (comprising three PNP, 17 disease controls and eight healthy controls) as it was withdrawn by the manufacturer and we were unable to source further kits for testing of the remaining 33 samples.

All four IIF assays were performed as per the manufacturer's instructions. For each assay, serum was diluted at 1:10 with PBS and added to the test slide. After incubation for 30 min and subsequent washing, polyclonal rabbit anti-human IgG (Dako, Denmark) was added and incubated for a further 30 min. IIF slides were read by two independent readers (SC, RS) blinded to the diagnosis, and each run included positive and negative controls. Any samples deemed 'equivocal' by one or both of the readers were arbitrated by a third independent reader (MWL). Examples of positive IIF results are shown in Fig. 1.

The envoplakin ELISA was performed as per manufacturer's instructions by one blinded operator. Briefly, serum samples were diluted 1:101 and added to microplate wells. After 30 min incubation, wells were washed and peroxidase-labelled anti-human IgG was added, followed by another 30 min incubation. After washing, bound conjugate was developed with chromogen. Results were deemed either positive or negative based on a defined cut-off value supplied by the manufacturer.

Statistical analysis

Standard statistical analysis to calculate sensitivity and specificity, along with 95% confidence intervals, was performed on the total control group ($n = 58$), and repeated separately within this group omitting normal control samples ($n = 48$).

RESULTS

IIF on either rat or monkey bladder substrate detected all three PNP patients, and interpretation of the fluorescence pattern was straightforward, with good inter-rater reproducibility (data not shown). Furthermore, in our cohort of 61 individuals, we found a high specificity for both assays (97% and 95%, respectively) for exclusion of PNP (Table 1).

The envoplakin ELISA was equally specific (98%), although only identified one of the three PNP sera. The CBA was found by both readers to be difficult to interpret, with many samples having equivocal staining, and was also less specific (82%). Lastly, IIF on rat cardiac tissue, whilst detecting two of the three PNP sera, had a higher rate of false positives, with a specificity of only 83%.

DISCUSSION

PNP can be a difficult diagnosis to establish since clinical and histological features overlap with other autoimmune blistering diseases.[4–7] Therefore, serological detection of pathogenic autoantibodies has an important diagnostic role, supported by their inclusion in a number of diagnostic criteria.[2,5,10] In applying such diagnostic criteria, it is essential to take into account methodological variations in assay performance, particularly with the release of commercial assays purporting to provide diagnostic equivalence. Here we have attempted to address some of these issues with a head-to-head comparison of traditional versus newer assays from a cohort gleaned from a

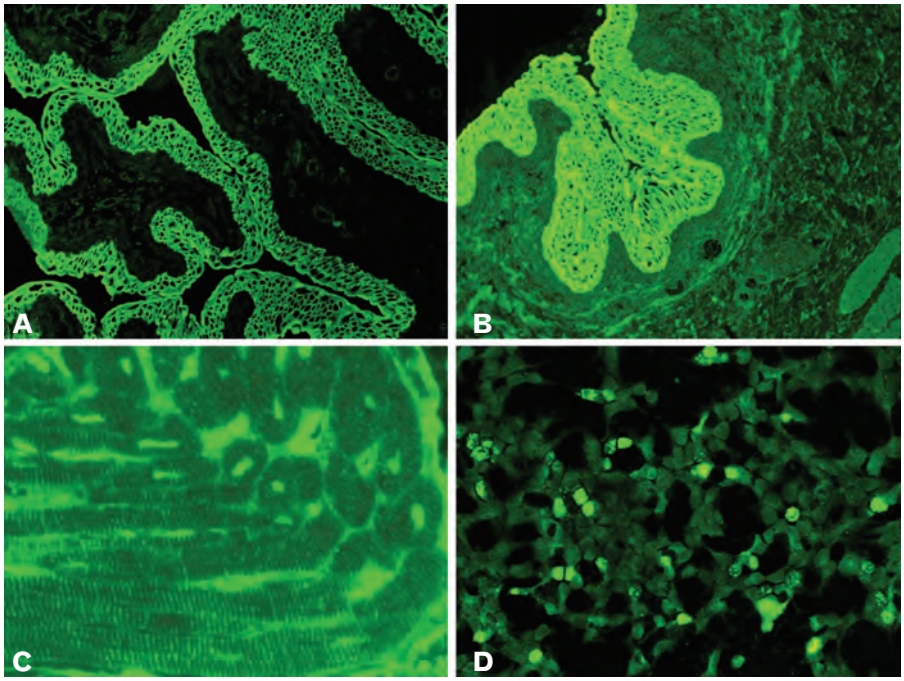

Fig. 1 IIF assays used in this study showing staining from a positive paraneoplastic pemphigus (PNP) sample (A–C) and a disease control sample (D). (A) Rat bladder and (B) monkey bladder, showing staining of the plakin-rich transitional epithelial cells; (C) rat cardiac tissue showing staining of the plakin-containing intercalated disks; (D) envoplakin-transfected HEK293 cells showing strong positive staining but from a patient who did not have PNP.