Detection of Laminin 5-Specific Auto-antibodies in Mucous Membrane and Bullous Pemphigoid Sera by ELISA

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Mucous membrane pemphigoid (MMP) is an autoimmune bullous disease that primarily affects mucous membranes leading to a scarring phenotype. MMP patients produce auto-antibodies (auto-ab) that preferentially recognize two components of the dermoepidermal basement membrane zone (BMZ): bullous pemphigoid (BP)180 and laminin 5 (LN5). Since detection of disease-specific auto-ab may be critical for diagnosis of MMP, we developed an ELISA with affinity-purified native human LN5. A total of 24 MMP, 72 BP, and 51 control sera were analyzed for LN5-specific auto-ab: 18/24 (75.0%) MMP and 29/72 (40.3%) BP sera were LN5 reactive. Sensitivity and specificity of the LN5 ELISA for MMP were 75% and 84.3%, respectively, and 40.3% and 88.2% for BP, respectively. The LN5 ELISA was more sensitive than a dot blot assay with native LN5, which detected LN5-reactive IgG in 14/24 (58.3%) MMP and 16/72 (22.2%) BP sera. In MMP, but not BP, levels of LN5-reactive IgG correlated with disease severity. Furthermore, IgG reactivity to LN5 of the MMP and BP sera was not significantly associated with IgG reactivity against other autoantigens of the BMZ, such as BP180 or BP230. Thus, the established LN5 ELISA holds great promise as a novel diagnostic and prognostic parameter for MMP.

Key words: BP180/dermoepidermal junction-auto-antibody/laminin 5/pemphigoid
J Invest Dermatol 124:732–740, 2005

Mucous membrane pemphigoid (MMP, formerly cicatricial pemphigoid) is a chronic autoimmune bullous disease that mainly affects mucous membranes, such as the conjunctivae, oral, and genital mucosa, as well as the perianal region, leading to scarring of the affected tissue (Bedane *et al*, 1997; Murakami *et al*, 1998). Cutaneous involvement is seen in approximately 20% of the patients and usually affects the head, neck, and upper trunk. Morbidity because of MMP may be high, depending on the affected regions. Ocular involvement bears the risk of symblepharon and ankyloblepharon, causing visual impairment or blindness and laryngeal stenosis may require tracheostomy. The frequency of laryngeal involvement in MMP is 8%–9% (Anhalt and Diaz, 1989; Ahmed *et al*, 1991; Nousari *et al*, 1999). Moreover, MMP has also been associated with internal malignancies (Egan and Yancey, 2000; Egan *et al*, 2001). Because of the risk of these severe complications, early recognition and aggressive treatment of MMP is essential.

Abbreviations: ab, antibody; BMZ, basement membrane zone; BP, bullous pemphigoid; CI, confidence interval; FN, fibronectin; IIF, indirect immunofluorescence; LN5, laminin 5; MMP, mucous membrane pemphigoid; OD, optical density; PBS, phosphate-buffered saline; ROC, receiver operating characteristic; RT, room temperature; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SSS, saline split skin; TBS, tris-buffered saline

Patients with MMP exhibit IgG and/or IgA auto-antibodies (ab) directed against heterogeneous components of the dermoepidermal basement membrane zone (BMZ) including bullous pemphigoid (BP)180, BP230 (Caproni *et al*, 2003), the 97/120 kDa LABD antigen, laminin 5 (LN5), LN6 (Domloge-Hultsch *et al*, 1992; Kirtschig *et al*, 1995; Ghohestani *et al*, 1996b; Chan *et al*, 1997; Bhol *et al*, 2000), and the integrin β4 subunit (Tyagi *et al*, 1996).

Among the various autoantigens of MMP, LN5 and BP180 are presumably recognized by the majority of the MMP sera. BP180 is a hemidesmosomal transmembrane type II protein with a large extracellular portion consisting of 15 interrupted collagenous subdomains located between a non-collagenous membrane-adjacent domain termed NC16A and a COOH-terminal domain termed NC1 region (Giudice *et al*, 1991; Hopkinson *et al*, 1992). It serves as a cell surface receptor (Giudice *et al*, 1992; Hopkinson *et al*, 1992), and plays an important role in the maintenance of epidermal–stromal adhesion (Borradori and Sonnenberg, 1999). Potential ligands of BP180 are a6β4 integrin and LN5, which have both been identified as autoantigens of MMP (Hopkinson *et al*, 1995; Borradori and Sonnenberg, 1999; Sonnenberg *et al*, 1999). Mutations within the BP180 gene (COL17A1) are the cause of a clinical variant of non-Herlitz junctional epidermolysis bullosa, a congenital disorder characterized by skin fragility and blistering (McGrath *et al*, 1995). In a passive transfer model, rabbit ab raised against

the murine homologue of the human NC16A domain of BP180 were able to induce subepidermal blister formation, reproducing all key features of BP (Liu *et al*, 1993). Furthermore, it has been shown that the serum levels of IgG auto-ab directed against the extracellular domain of BP180 are related to the severity of BP (Dopp *et al*, 2000; Hofmann *et al*, 2002; Thoma-Uszynski *et al*, 2004). It is noteworthy that IgG auto-ab against the COOH-terminal region of the BP180 ectodomain seem to be associated with the characteristic features of MMP (Nakatani *et al*, 1998; Lazarova *et al*, 2000).

LN5 is a multifunctional glycoprotein of the BMZ that plays an important role in the initiation and maintenance of epithelial cell anchorage to the underlying connective tissue. LN5, originally termed nicein (Verrando *et al*, 1987), kalinin (from greek $\chi\alpha\lambda\iota\nu o\varsigma$ = thong or bridle) (Rousselle *et al*, 1991; Burgeson *et al*, 1994), and epiligrin (Carter *et al*, 1991; Domloge-Hultsch *et al*, 1992) has been identified as a heterotrimeric glycoprotein consisting of α3, β3, and γ2 subunits that are covalently linked by disulfide bonds. LN5 is produced both by epithelial and mesenchymal cells and is abundantly expressed in the skin, trachea, esophagus, cornea, amnion, and intestinal smooth muscle (Rousselle *et al*, 1991), all of which possess hemidesmosomes. It binds to $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrins, with the latter being crucial for hemidesmosome assembly. LN5 is initially synthesized in a cell-associated form estimated at 460 kDa. The cellular form contains non-identical subunits of 200–165, 140, 155–105–80 kDa linked by interchain disulfide bonds (Marinkovich *et al*, 1992; Jones *et al*, 1998). Gene defects of the $\alpha 3\beta 3\gamma 2$ chains are the cause of Herlitz and non-Herlitz junctional epidermolysis bullosa (Pulkkinen *et al*, 1994, 1995; Vailly *et al*, 1995). The passive transfer of LN5-reactive IgG from sera of patients with MMP into neonatal mice reproduces the pathology with a subepidermal loss of adhesion and the appearance of tense blisters (Lazarova *et al*, 1996). All three chains of LN5 have been shown to be recognized by LN5-reactive MMP sera and recent studies utilizing bacteria-derived recombinant proteins suggested that most MMP sera recognize the G domain of the α3 subunit (Hisamatsu *et al*, 2003).

The aim of this study was to develop a highly sensitive and specific ELISA for the detection of LN5-reactive IgG in a cohort of MMP and BP sera including a control group. There is a need to develop new diagnostic tools to identify patients with MMP with IgG auto-ab against LN5 to prevent long-term complications of the disease, which is associated with a late onset of immunosuppressive therapy. Our data demonstrate that IgG auto-ab against LN5 are not only present in the majority of MMP sera but also, at lower levels, in a substantial number of BP sera. Moreover, the IgG titers against LN5 seem to reflect disease activity and may thus provide a critical prognostic marker for MMP.

Results

Characterization of the patients' sera by indirect immunofluorescence (IIF) Sera from patients with the clinical diagnosis of MMP or BP were subjected to IIF analysis utilizing saline-split skin (SSS). Among the MMP sera, 8/24 (33.3%) showed a dermal, 2/24 (8.3%) an epidermal pattern, and 1/24 (4.2%) a combined epidermal/dermal pattern whereas 13/24 sera (54.1%) were negative. In contrast, the majority, i.e. 58/72 (80.5%) of the BP sera, showed IgG reactivity with the epidermal side of the SSS whereas 4/72 (5.5%) reacted with the dermal side of SSS and 2/72 BP sera showed a combined SSS staining pattern. A total of 8/72 (11.1%) BP sera were negative by SSS analysis.

Affinity-purified LN5 is recognized by ab directed against the LN α3, β3, and γ2 chains The quality of the purified LN5 was evaluated by silver staining of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (Fig 1*A*) and immunoblot analysis (Fig 1*B*). By western blot, specific bands of processed LN5 were detected at 165, 140, 155–105 kDa corresponding to the LN α3, β3, and γ2 chains. Thus, affinity purification led to substantial amounts of intact and fully immunoreactive LN5.

Detection of LN5-specific IgG in MMP and BP sera by ELISA Sera from patients with MMP (n = 24), BP (n = 72), and from age-matched controls (n = 51) were tested by ELISA against native LN5, recombinant BP180, BP230, and fibronectin (FN). Based on the maximization of the Youden index, the cut-off point for the ELISA with LN5 protein was set at 0.219 optical density (OD) units for the MMP sera, which corresponds to a Youden index of 0.563 (Fig 2*A*). Reactivity to LN5 was considered positive when the OD of the BP sera exceeded 0.230 units (Youden index of 0.285) (Fig 2*B*).

The diagnostic performance of each test was illustrated by receiver operating characteristic (ROC) curves (Fig 2*A* and *B*). Using the selected cut-off values, the sensitivity of the LN5 assay with the MMP sera was 75.0% (95% confidence interval (CI): 53.3%–90.2%) and the specificity was

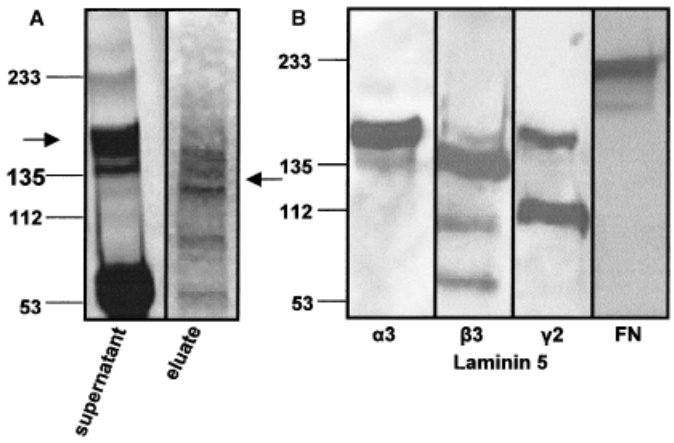

Figure 1
(*A*, *B*) Affinity-purified laminin 5 (LN5). (*A*) Silver stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels run under reducing conditions of LN5 from SCC-25 cell culture media, before and after affinity purification from culture supernatants. (*B*) Mouse anti-LN α3, goat anti-LN β3, and mouse anti-LN γ2, all showed reactivity with the purified LN5 preparation. The bands shown represent the normal unprocessed and processed forms of the LN5 chains. α3 chain, processed at 165 kDa; β3 chain, unprocessed at 140 kDa; and γ2 chain, unprocessed at 155 kDa and processed at 105 kDa. The content of residual fibronectin (FN) in the LN5 preparation was visualized by a goat anti-FN ab. Numbers on the left of the gels indicate migration positions of molecular weight marker.