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
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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

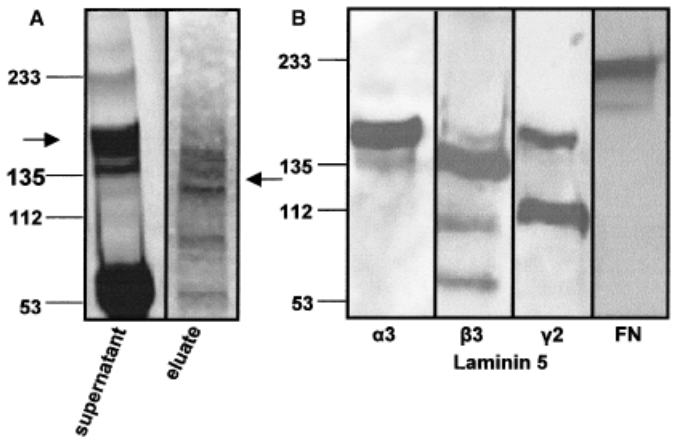


Figure 1
(*A*, *B*) Affinity-purified laminin 5 (LN5). (*A*) Silver stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels run under reducing conditions of LN5 from SCC-25 cell culture media, before and after affinity purification from culture supernatants. (*B*) Mouse anti-LN α3, goat 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.

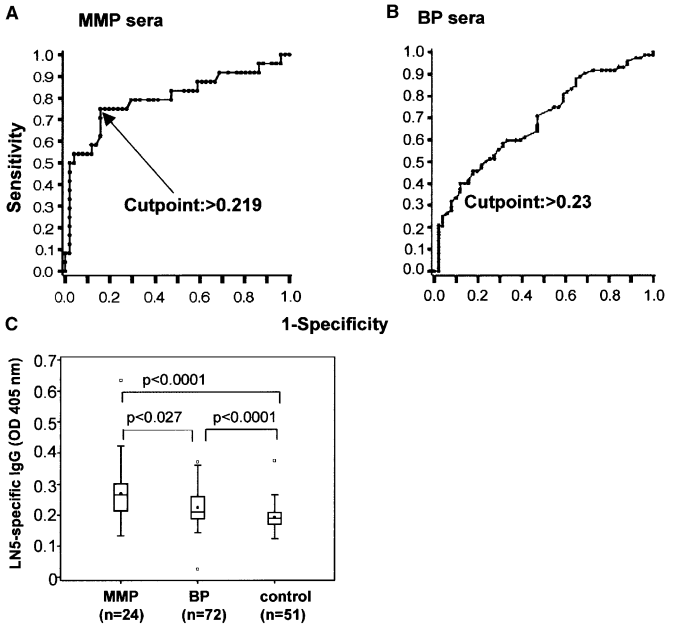


Figure 2
***(A–C)* Sensitivity and specificity of the laminin 5 (LN5) ELISA.** The diagnostic properties of the LN5 ELISA are depicted as receiver operating characteristic curves for (*A*) mucous membrane pemphigoid (MMP) and (*B*) bullous pemphigoid (BP) sera. Sera of patients with active MMP (n = 24), BP (n = 72), and controls (n = 51) were screened for LN5 IgG reactivity by ELISA. (*C*) Comparing the IgG reactivity against LN5 in the MMP, BP, and control sera, a significant difference in the LN5 IgG autoantibody status was found between all three groups in pairwise tests.

84.3% (95% CI: 71.4%–93.0%), whereas in the BP sera, the sensitivity was 40.3% (95% CI: 28.9%–52.5%) and the specificity was 88.2% (95% CI: 76.1%–95.6%). The diagnostic odds ratio was 16.1 (95% CI: 4.9–53.2) in case of MMP and 5.1 (95% CI: 1.9–13.4) for BP (Table I).

Based on this diagnostic classification, 18/24 (75.0%, 95% CI: 53.3%–90.2%) MMP and 29/72 (40.3%, 95% CI: 28.9–52.5) BP sera were LN5 reactive (Fig 2*C*). Statistically significant differences in anti-LN5 IgG reactivity were observed between MMP, BP, and control sera (Kruskal–Wallis test: $p < 0.0001$, 95% CI: 0–0.0005). In pairwise *post hoc* Wilcoxon's tests, the difference between OD values of MMP or BP patients and controls was highly significant. Furthermore, the difference between MMP and BP OD values ($p = 0.027$, 99% CI: 0.023–0.032) was significant. Addressing the dichotomized outcome, IgG reactivity against LN5 was significantly more often found in MMP sera than in BP sera (Fisher's exact test, two sided: $p = 0.0044$) (Fig 2*C*).

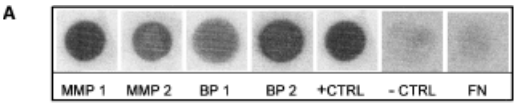
In order to exclude background reactivity to FN, the major contaminant of the native LN5 preparation, we tested each serum sample against FN by ELISA and dot blot analysis. We found a low, negligible reactivity of the tested MMP and BP sera against FN (data not shown). Comparison of the LN5 and FN OD values by ELISA revealed only weak to moderate, albeit significant correlation between LN5 and FN activity (Spearman's correlation coefficient 0.453, $p < 0.0001$). Similar results were also shown by dot blot assay.

Table I. Sensitivity and specificity of the laminin 5 (LN5) ELISA

	MMP (n = 24)	BP (n = 72)
Cutpoint	>0.219	>0.230
LN5 positive (n)	18	29
Sensitivity (exact 95% CI)	75.0% (53.3–90.2)	40.3% (28.9–52.5)
Specifity (exact 95% CI)	84.3% (71.4–93.0)	88.2% (76.1–95.6)
Youden index	0.593	0.285
Diagnostic odds ratio[a] (95% CI)	16.1 (4.9–53.2)	5.1 (1.9–13.4)
Positive predictive value (exact 95% CI)	18/26 69.2% (48.2–85.7)	29/35 82.9% (66.4–93.4)
Negative predictive value (exact 95% CI)	43/50 87.8% (75.2–95.4)	45/88 51.1% (40.3–62.0)

MMP, mucous membrane pemphigoid, BP, bullous pemphigoid; CI, confidence interval.
[a](true positives/false negatives)/(false positives/true negatives).

Detection of LN5-reactive IgG by ELISA and dot blot analysis A total of 14/24 MMP (58.3%) and 16/72 BP (22.2%) sera yielded positive results in the dot blot assay with LN5 (Fig 3*A* and *B*). Of the 14 LN5-responsive MMP patients, five (35.7%) suffered from extensive, five (35.7%) from limited skin lesions, and four (28.6%) from exclusive mucosal involvement. Overall, mucosal involvement was seen in 13 (92.9%) MMP patients. Among the 16 LN5-reactive BP patients, 13 (87.3%) had extensive, three (18.8%) limited skin lesions, and three (18.8%) only showed mucosal involvement. The details of the clinical phenotype of the MMP and BP patients are shown in Fig 3*B*. Both LN5-reactive MMP and BP sera were more frequently positive by LN5 dot blot analysis than the LN5-negative sera (Table II). Specifically, 11/18 MMP (61.1%) and 11/29 BP (37.9%) sera that were LN5 reactive by ELISA were also positive by dot blot assay. In general, the diagnostic power of the LN5 ELISA was clearly beyond that of the LN5 dot blot assay even though there were 3/24 (12.5%) MMP and 5/72 (6.94%) BP sera that were found to be LN5 reactive by dot blot but not by ELISA (Table II). When FN was used as



B

Dot Blot	Clinical symptoms								
Dot blot reactivity	Skin* extensive	Skin limited	Mucosal involv.	oral	genital	conjuctival	capillitium	atrophy	milia
MMP n=14 (58.3%)	5 (35.7%)	5 (37.5%)	13 (92.9%)	8 (57.1%)	3 (21.4%)	5 (36.0%)	3 (21.4%)	3 (21.4%)	1 (7.1%)
BP n=16 (22.2%)	13 (87.3%)	3 (18.8%)	3 (18.8%)	3 (18.8%)	1 (6.25%)	0	0	0	0

Figure 3
***(A, B)* Anti-laminin 5 (LN5) reactivity of the mucous membrane pemphigoid (MMP) and bullous pemphigoid (BP) sera by dot blot analysis.** (*A*) A total of 24 MMP, 72 BP, and 51 control sera were analyzed for LN5 IgG reactivity by dot blot. 14/24 (58.3%) MMP and 16/72 (22.2%) BP sera reacted with LN5 by dot blot analysis. Representative dots of the LN5-reactive patients' and control sera are shown. Fibronectin (FN) was used as a control protein. (*B*) The clinical phenotype of the LN5-reactive MMP and BP patients is depicted.

Table II. Diagnostic power of ELISA and dot blot assay in the detection of IgG reactive with laminin 5 (LN5) in mucous membrane pemphigoid (MMP) and bullous pemphigoid (BP) sera

	LN5 dot blot	LN5 ELISA		
		Positive	Negative	Total
MMP sera (n = 24)	Positive	11 (45.0%)	3 (12.5%)	14 (58.3%)
	Negative	7 (29.0%)	3 (12.5%)	
BP sera (n = 72)	Positive	11 (15.3%)	6 (8.3%)	16 (22.2%)
	Negative	18 (25.0%)	38 (52.7%)	

a control antigen in the LN5 ELISA and dot blot analyses, the MMP, BP, and control sera showed only marginal IgG reactivity (not shown) (Fig 4).

LN5-specific IgG reactivity and correlation with clinical severity of MMP and BP Among the patients studied, 10/24 (41.7%) MMP and 20/72 (27.8%) BP patients presented with a limited skin involvement, whereas 8/24 (33.3%) MMP and 50/72 (69.4%) BP patients showed extensive skin lesions. The remaining MMP (n = 1) and two BP patients (n = 2) were in remission (Table III). Sera from MMP patients with extensive skin involvement showed higher average LN5-specific IgG auto-ab levels by ELISA ($p = 0.044$, 99% CI: 0.039–0.05 (Kruskal–Wallis test for overall homogeneity)) than patients with limited disease (Fig 5*A*). Pairwise *post hoc* statistical testing revealed that the difference between limited and extensive skin involvement in the MMP patients was significant ($p = 0.021$, 99% CI: 0.017–0.025). In contrast, in the BP sera, the IgG auto-ab levels against LN5 neither showed an association with disease severity nor with the extent of mucosal involvement (Fig 5*B*). Moreover, there was no significant difference in the titers of anti-LN5 IgG between untreated and treated MMP and BP patients. The direct impact of immunosuppressive treatment on IgG auto-ab titers in individual patients needs to be evaluated in a larger cohort.

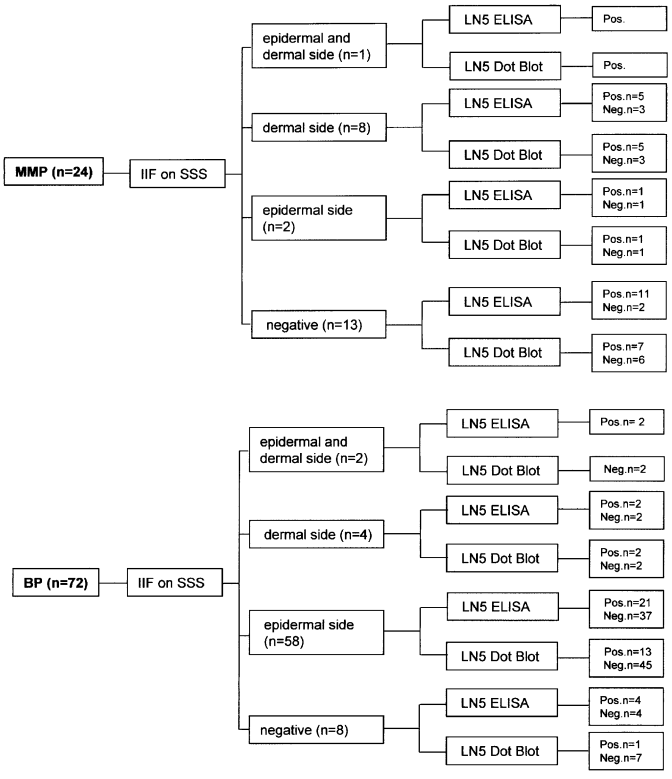


Figure 4
Immunoserological profile of mucous membrane pemphigoid (MMP) and bullous pemphigoid (BP) patients. The IgG reactivity pattern of MMP and BP sera with saline-split human skin (SSS) compared with IgG reactivity to laminin 5 (LN5) by ELISA and dot blot analysis are shown. A significant portion of the 13 SSS-negative MMP sera and eight SSS-negative BP sera were reactive for LN5 by ELISA or dot blot analysis.

IgG levels against LN5 relate to clinical severity in a patient with MMP To further substantiate the findings with the MMP sera, a patient with extensive MMP was subjected to horizontal serological analysis (Fig 6). This patient initially presented with bullous and erosive lesions of the oral mucosa, conjunctivae, and extensive skin involvement with tense blisters that healed with milia formation and atrophy on the trunk, capillitium, conjuctivae, oral, and genital mucosa (Fig 6*A*). Serum samples taken over a period of 120 wk were tested for LN5-specific IgG by ELISA (Fig 6*B*) and by dot blot analysis (Fig 6*C*). LN5-specific IgG titers were maximal when extensive skin and mucous membrane involvement was present and slowly declined upon clinical improvement after administration of immunsuppressive treatment with high-dose methylprednisolone, intravenous immunoglobulins, and mycophenolate mofetil. Thus, the levels of circulating LN5-specific IgG auto-ab seemed to relate to the extent of the overall clinical disease activity in this case of extensive MMP. A strict direct correlation between the extent of cutaneous and/or mucosal lesions, however, was hard to establish. It is noteworthy that ELISA values and findings of dot blot analysis correlated well (Fig 6).

The presence of LN5-specific IgG does not relate to IgG reactivity for BP180 or BP230 IgG reactivity against LN5 of the MMP and BP sera was also compared with anti-BP180 and anti-BP230 IgG reactivity. A total of 6/24 (25.0%) MMP and 55/72 (76.4%) BP sera showed IgG reactivity against BP180, whereas IgG reactivity against BP230 was observed only in 2/24 (8.3%) MMP and 34/72 (47.2%) BP sera. It is noteworthy that 4/24 (16.7%) MMP and 8/72 (11.1%) BP sera did not show reactivity either to BP180 and/or BP230 (Table IV).

Among the 18 LN5-reactive MMP sera, four (22.2%) showed IgG reactivity for BP180 and two (11.1%) for BP230. Of the 29 LN5-reactive BP sera, 22 (75.9%) showed IgG reactivity to BP180 and 12 (41.4%) to BP230. In contrast, only 22/55 (40.0%) of the BP180-reactive BP sera also showed IgG reactivity to LN5.

Taken together, there was no significant association between LN5-specific IgG and IgG reactivity for BP180 or BP230 both in the MMP and BP sera (Fig 7*A* and *B*). Remarkably, the sera from two MMP patients with extensive

Table III. Clinical phenotype of studied mucous membrane pemphigoid (MMP) and bullous pemphigoid (BP) patients[a]

	Clinical involvement								
	Skin extensive[b]	Skin limited[c]	Mucosal involvement	Oral	Genital	Conjunctival	Capillitium	Atrophy	Milia
MMP, n = 24	8 (33.3%)	10 (41.7%)	20 (83.3%)	16 (66.7%)	4 (16.7%)	9 (37.5%)	6 (25%)	10 (41.7%)	1 (4.2%)
BP, n = 72	50 (69.4%)	20 (27.8%)	11 (15.3%)	9 (12.5%)	2 (2.7%)	1 (1.4%)	2 (2.7%)	0	0

[a]One MMP and two BP patients were in remission.
[b]Extensive disease, involvement of >15% of total body surface.
[c]Limited disease, involvement of <15% of total body surface.

mucous membrane involvement, who showed a strong IgG response to LN5 by ELISA and dot blot analysis, were also reactive to BP230 (not shown).

Discussion

This study was designed to assess the auto-ab reactivity of MMP sera against LN5 by a novel ELISA. Our data demonstrate that the majority (75%) of the MMP sera contained LN5-specific IgG. These findings support the concept that LN5 is a major auto-ag of MMP. The LN5-negative MMP sera were presumably reactive to other components of the BMZ as shown for some of the sera that were BP180 responsive. It is noteworthy that 40.3% of the sera from patients with BP, a related autoimmune bullous disorder, also exhibited titers for LN5-specific IgG above the diagnostic threshold chosen. Moreover, the titers of anti-LN5 IgG were found to reflect disease severity in the MMP patients, but not in patients with BP. The detection of LN5-specific IgG was relatively specific since control sera from patients with unrelated skin conditions were only exceptionally reactive. In this study, dot blot analysis was used as a complementary assay to confirm the results obtained by ELISA. This method presented lower sensitivity but better specificity with regard to making diagnosis in MMP compared with the ELISA (Table III).

The ELISA protocol presented in this report provides a convenient and sensitive means by which LN5-specific IgG auto-ab can not only be detected but also quantified. These findings strongly suggest (i) that the LN5 ELISA is highly sensitive and specific for MMP, (ii) that the ELISA also has a prognostic value in MMP, and (iii) that auto-ab against LN5 are not restricted to MMP, but are also present in BP.

Previous reports on LN5-specific auto-ab in autoimmune blistering diseases of the pemphigoid group were based on immunoblot and immunoprecipitation analyses utilizing epidermal extracts (Kirtschig *et al*, 1995; Ghohestani *et al*, 1997; Hsu *et al*, 1997; Nousari *et al*, 1999). In these studies, native LN5 was isolated from the extracellular matrix of cultured human keratinocytes for immunoblotting and/or immunoprecipitation of MMP serum samples and controls. There was variability in the data obtained with immunoprecipitation experiments ranging from values of 37% to 100% of MMP patients having a titer for LN5 (Domloge-Hultsch *et al*, 1992; Kirtschig *et al*, 1995; Shimizu *et al*, 1995; Hashimoto *et al*, 1996; Lazarova and Yancey, 1996; Chan *et al*, 1997), whereas in the case of immunoblotting experiments

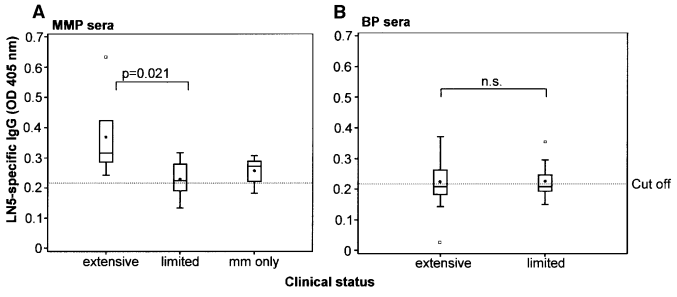


Figure 5
(*A*, *B*) IgG titers against laminin 5 (LN5) correlate with the clinical severity of mucous membrane pemphigoid (MMP) but not of bullous pemphigoid (BP). (*A*) MMP patients with extensive skin involvement had higher LN5-specific IgG autoantibody levels than MMP patients with limited or exclusive mucous membrane involvement. (*B*) In contrast, anti-LN5 IgG reactivity of the BP sera did not show such a relationship with the clinical status (ns, not significant).

A

Observation period (weeks)						
Symptoms	4	28	30	34	88	120
Skin	lim.	ext.	lim.	ext.	none	none
Capillitium	+	+	+	+	-	-
Ocular involvement	+	+	+	+	+	+
Oral mucosa	+	+	+	+	+	-
Genital mucosa	+	-	-	-	-	-
Atrophy	-	+	+	+	+	+
Milia	-	+	+	+	+	+

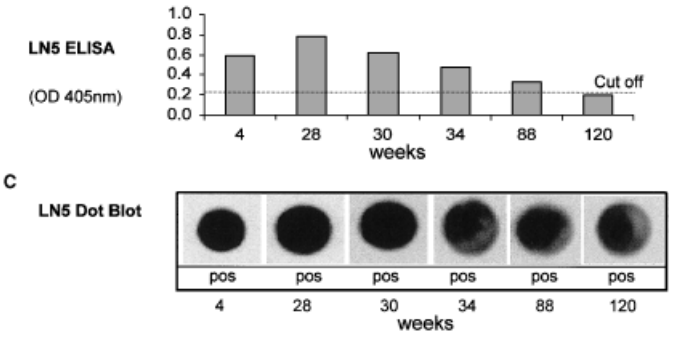


Figure 6
(*A–C*) Anti-laminin 5 (LN5) IgG titers correlate with disease activity in a patient with mucous membrane pemphigoid (MMP). The clinical phenotype of a MMP patient (*A*) over a course of 120 wk and corresponding serum IgG reactivity to LN5 by ELISA (*B*) and by dot blot analysis (*C*) are shown. Remarkably, upon clinical remission the titers of LN5-reactive IgG declined to threshold levels.

Table IV. Autoantibody profile of mucous membrane pemphigoid (MMP) and bullous (BP) pemphigoid sera by ELISA with native laminin 5 (LN5) and recombinant BP180 and BP230

LN5[a]	BP180[b]	BP230[c]	MMP sera (n = 24)	BP sera (n = 72)
+			18 (75.0%)	29 (40.3%)
	+		6 (25.0%)	55 (76.4%)
		+	2 (8.3%)	34 (47.2%)
	+	+	1 (4.2%)	30 (41.7%)
+	+		4 (16.7%)	22 (30.6%)
+		+	2 (8.3%)	12 (16.7%)
+	+	+	1 (4.2%)	12 (16.7%)
−			6 (25.0%)	43 (59.7%)
−	+		2 (8.3%)	33 (45.9%)
−		+	0 (0%)	22 (30.6%)
−	+	+	0 (0%)	19 (26.4%)
−	−	−	4[d] (16.7%)	8 (11.1%)

[a]LN5 purified from SCC-25 cell culture supernatant.
[b]BP180, entire extracellular domain of BP180 (baculovirus derived).
[c]BP230, NH_2, and COOH terminus of BP230 (baculovirus derived).
[d]These four MMP sera were also unresponsive to collagen VII.
Gray bars; autoantigen was not taken into consideration.
−, not reactive; +, reactive to above antigen.

between 26% and 83% of the MMP sera were found to have an LN5 IgG titer (Ghohestani *et al*, 1996a; Kirtschig, 1998; Nousari *et al*, 1999; Schmidt *et al*, 2001; Hisamatsu *et al*, 2003).

Immunoprecipitation seems to outperform immunoblot with regard to sensitivity (Hisamatsu *et al*, 2003). Possible explanations include differences in selection and number of the sera investigated as well as varying sensitivities and specificities depending on the detecting system applied. Furthermore, by immunoprecipitation native LN5 is detected, whereas by immunoblot denatured LN5 is recognized by the sera. Thus, it must be considered that the sensitivity of the immunoblot may be influenced by loss of immunoreactivity of antigenic epitopes because of partial denaturation of LN5 by SDS gel electrophoresis. A previous study suggested that IgG reactivity against LN5 was detected both under reducing and non-reducing conditions (Chan *et al*, 1997).

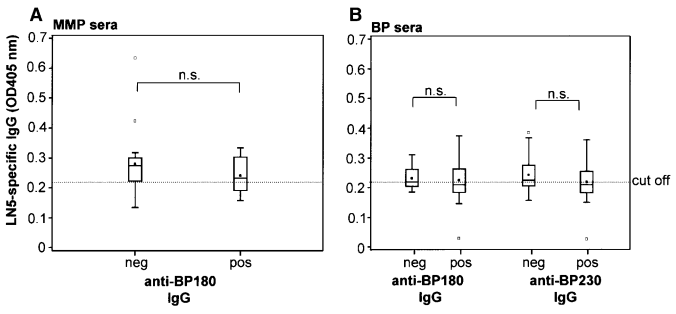


Figure 7
***(A, B)* Relationship of IgG reactivity for laminin 5 (LN5) and bullous pemphigoid (BP)180 or BP230 of mucous membrane (MMP) and BP sera.** A total of 6/24 (25.0%) of the MMP sera were reactive to BP180, four of which (16.7%) showed IgG reactivity to LN5 (*A*). Among the BP sera, 55/72 (76.4%) were positive for BP180 and 34/72 (47.2%) for BP230. A total of 22/72 (30.6%) of the BP180-reactive BP sera were also LN5 reactive and 12/72 (16.7%) of the BP230-positive BP sera showed IgG reactivity to LN5 (*B*) (ns, not significant).

A considerable portion of the LN5-responsive MMP and BP sera were negative by IIF with human SSS as a substrate. This finding is not novel in light of previous studies that showed that BP and MMP sera that were negative by IIF were at times reactive with dermoepidermal basement membrane proteins by immunoblot analysis (Ghohestani *et al*, 1996a, 1997). In this study, 4/8 IIF-negative BP sera showed only low titered IgG auto-ab against LN5 by ELISA, which may explain the differential reactivity. In contrast, 11 IIF-negative MMP sera that were LN5 responsive by ELISA had higher auto-ab titers than the BP sera. The lower sensitivity of IIF may be explained by a lower concentration of antigenic epitopes of LN5 on the substrate that may be more susceptible to proteolytic degradation. Purified LN5 utilized in a "native" form by ELISA was also superior to the identical denatured LN5 of immunoblot analysis. Thus, to definitively rule out the clinical suspicion of MMP, the present ELISA should be used as an additional diagnostic tool.

Several cellular sources, including normal human keratinocytes (Hsu *et al*, 1997), the rat cell line 804G (Baker *et al*, 1996), the human MCF-10A cell line (Stahl *et al*, 1997), the human SCC-25 cell line (Rousselle and Aumailley, 1994), and the SCC-12 human cell line (Plopper *et al*, 1996), were utilized as LN5 sources in the past. An LN5 ELISA with extracts from cultured human keratinocytes was used to determine the IgG subclass distribution in selected LN5-reactive sera (Hsu *et al*, 1997). It is noteworthy that in this study, the majority of the MMP sera was negative by IIF, but LN5 responsive by immunoprecipitation. In addition, an LN5 ELISA using 804G cell-conditioned medium was used for cell matrix investigations (Plopper *et al*, 1996). Moreover, an LN5 ELISA was introduced for the quantification and characterization of LN5 synthesized by human keratinocytes (Amano *et al*, 1999).

Since LN5 is presumably a natural ligand of BP180, LN5-specific auto-ab may contribute to the pathogenesis of MMP by interfering with LN5–BP180 interaction resulting in loss of dermo-epidermal adhesion (Borradori and Sonnenberg, 1999; Nievers *et al*, 2000). This is in line with a previous report that auto-ab against the COOH terminus of BP180 contribute to the scarring phenotype observed in MMP patients (Balding *et al*, 1996). In this study, IgG reactivity against the COOH terminus of BP180 was detected in 61% of 23 MMP sera. The COOH-terminal region of BP180 has been identified as a major epitope recognized by MMP sera (Balding *et al*, 1996; Bedane *et al*, 1997; Nakatani *et al*, 1998). In two independent studies, the majority (16/19) of the MMP sera preferentially recognized the NH2-terminal NC16A region of the BP180 ectodomain (Schmidt *et al*, 2001), whereas in the second 7/10 (70%) MMP sera reacted with the NC16A domain of BP180 (Kromminga *et al*, 2002). Thus, MMP sera may react with one or both of these antigenic sites of BP180 (Balding *et al*, 1996). The coincidence of anti-LN5 and anti-BP180 (NC16A) IgG reactivity has also been reported by Kawahara *et al* (1998). In this study, 25%

of the MMP and BP sera showed dual IgG reactivity against BP180 and LN5 but there was no direct correlation to IgG reactivity against LN5. None of the investigated BP patients experienced scarring formation of either skin or mucous membranes, which is in line with a previous ELISA study (Nakatani *et al*, 1998). According to a recent study by our group, 9/16 (52%) BP patients with extensive mucous membrane involvement showed a dual IgG response against the NH2- and COOH-terminal domains of BP180 (Hofmann *et al*, 2002).

In conclusion, the present ELISA with native LN5 is sensitive and specific in detecting LN5-reactive IgG not only in MMP but also in a subgroup of patients with BP. These findings suggest that LN5 is a major autoantigen of MMP. This novel ELISA assay holds great promise as a powerful diagnostic and prognostic tool in MMP and may eventually help to clarify the role that ab against LN5 play in the pathogenensis of MMP.

Materials and Methods

Patients and controls In this study, we investigated the sera of 24 MMP and 72 BP patients and 51 control donors. The clinical diagnosis of MMP with subepidermal blisters and/or signs of predominating mucous membrane involvement with dermal scarring was confirmed by direct IF (DIF) with IgG and/or C3 deposits at the dermal–epidermal BMZ of perilesional skin and/or circulating IgG binding to the dermal or epidermal side of 1M SSS by IIF microscopy. The diagnosis of BP was based on tense blisters on normal or erythematous skin, IgG and /or C3 deposits at the dermal–epidermal junction in perilesional skin by DIF, and/or circulating IgG binding to the epidermal side of SSS by IIF. All MMP patients suffered from mucosal lesions at one or more sites at the onset of the disease or at later stages (e.g., conjunctivae, oral, and genitoanal region). In contrast, mucosal involvement was only seen in 11/72 (15.3%) BP patients (Table I).

Disease severity of MMP and BP patients was scored according to the total skin area afflicted: (1) Limited disease was defined as the presence of few bullae distributed on a maximum of 15% of total body surface, (2) extensive disease was associated with bullae that involved a total skin area greater than 15%, and (3) patients in remission had experienced neither skin nor mucosal lesions for more than 1 mo. Several patients were on immunosuppressive treatment.

Control sera were also obtained from patients with pemphigus vulgaris (PV) ($n = 9$), pemphigus foliaceus ($n = 3$), and individuals ($n = 39$) who suffered from unrelated acute skin conditions such as herpes zoster, erysipelas, atopic dermatitis, or acute allergic conditions. The study was conducted according to the declaration of Helsinki principles. All patients and control donors gave informed written consent to participate in this study. Patients' and control sera were stored at $-20^{\circ}C$ prior to analysis. One serum from a patient with PV and two sera from unhealthy control individuals, which were available in large amounts, were used as negative reference sera for the LN5 IgG ELISA throughout the study.

Production and purification of LN5 The squamous carcinoma cell line SCC-25 (a generous gift from P. Rousselle) was cultured in 50% Ham's F-12 medium and 50% Dulbecco's-modified Eagle's medium (Gibco-Invitrogen, Karlsruhe, Germany) supplemented with 10% FCS (PAA, Cölbe, Germany), 2 mM glutamine (Sigma, Taufkirchen, Germany), hydrocortisone (0.4 μg per mL), and penicillin/streptomycin (Sigma) at 37°C in a humidified environment at 5% CO_2. Cells were harvested by washing subconfluent cultures with PBS, and with 5 mM EDTA/PBS, followed by incubation with trypsin/EDTA (Sigma) for 3–4 min at 37°C. Cells were, subsequently, resuspended in culture medium containing 10% FCS to neutralize trypsin, centrifuged at 1200 rpm (241 g) and resuspended in fresh culture medium. Cell cultures were subcultured every second day to maintain optimal culture conditions.

For LN5 purification, conditioned media from 80% subconfluent cultures of SCC-25 cells were collected, centrifuged (to remove cell debris), sterile filtered, and stored at 4°C after addition of protease inhibitors (5 mM EDTA, 50 μM phenylmethylsulfonylfluoride and 50 μM *N*-ethylmaleimide; all from Sigma).

LN5 was purified by affinity chromatography from conditioned medium as follows: first, FN was removed by chromatography over gelatin sepharose (20 mL bed volume; Amersham, Freiburg, Germany), which was equilibrated in 50 mM Tris-HCl at pH 8. Subsequently, LN5 was purified by passage over BM165 sepharose (monoclonal anti-LN5 ab BM165, provided by P. Rousselle) (Rousselle *et al*, 1991). LN5 was eluted from BM165 sepharose using 0.1 M triethylamine. The fractions containing LN5 were pooled, neutralized by adding 1 M Tris-HCl, pH 8, finally dialyzed against PBS, and stored at 4°C. The eluted protein fraction revealed the 200–165, 140, 155–105–80 kDa bands corresponding to the unprocessed and processed forms of the α3, β3, and γ2 chains of LN5 as shown in Fig 1(Rousselle *et al*, 1991; Jones *et al*, 1998; Goldfinger *et al*, 1998). Protein concentrations were determined according to a modified protocol by bicinchoninic acid assay (Pierce, Bonn, Germany).

Gel electrophoresis and immunoblot analysis of LN5 LN5 was separated by a SDS-PAGE under reducing conditions (Laemmli, 1970) and was visualized either by silver-stain (Bio-Rad, Munich, Germany) or transferred onto nitrocellulose (Bio-Rad) for immunoblot analysis according to standard procedures (Hofmann *et al*, 2002). Protein samples were diluted in 5 × sample buffer containing 10% SDS, 50% glycine, 10% 2-β-mercaptoethanol, and 0.1% bromophenol blue, denatured for 3 min at 100°C, and separated by SDS-PAGE on 5% polyacrylamide gels. Separated proteins were transferred onto nitrocellulose (Bio-Rad) using a transblot system (Biometra, Göttingen, Germany). The membranes were cut into strips and blocked with milk buffer (5% non-fat dried milk in Tris-buffered saline (TBS)/0.15% Tween 20) for 1 h. Ab used included mouse-anti-LNα3 at 1:1000 (BM165, kindly provided by P. Rousselle), goat-anti-LNβ3 at 1:500 (Santa Cruz, Heidelberg, Germany), mouse-anti-LNγ2 at 1:1000 (Chemicon, Hofheim, Germany), and goat-anti-FN at 1:500 (Serotec, Düsseldorf, Germany). All ab were diluted in milk buffer and incubates were performed overnight at 4°C. Ab coupled to horseradish peroxidase (HRP) were diluted at 1:10000 in milk buffer and added for 1 h at room temperature (RT). Immunoblots were washed 5 × in TBS and bound ab were visualized with anti-mouse (Dako, Hamburg, Germany), or anti-goat (Amersham) IgG diluted in TBS/0.15% Tween 20, and finally developed using ECL (Amersham).

LN5 ELISA Optimal conditions for the ELISA were established by testing various sera titrations following published guidelines (Kemeny, 1991). Microtiter 96-well plates (Maxisorb Immunoplate; Nunc, Wiesbaden, Germany) were coated overnight at 4°C with 1.5 μg LN5 per well. Wells were blocked with milk buffer for 30 min at RT. All sera were diluted at 1:50 and incubated for 1 h at RT with the LN5-coated ELISA plates. After three washes with milk buffer, the plates were incubated with HRP-conjugated anti-human IgG (Dako) for 1 h at RT. After washing, ab binding was visualized by adding acino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid (CalBiochem, Bad Soden, Germany) for 30 min at RT. The substrate reaction was measured at 405 nm by a Wallac 1420 microplate reader (Wallac, Freiburg, Germany). The cut-off values of the ELISA for the MMP sera (0.219 OD units) and for the BP sera (0.23 OD units) were calculated data adaptively based on ROC curves (Fig 2*A* and *B*).

To evaluate potential plate-to-plate variability, each plate included a positive control serum from a patient with extensive MMP and three sera from unhealthy control donors as negative controls. The values for the positive and negative controls did not

vary more than 10% when assays performed on different days were compared.

Since FN represented the major contaminant of the LN5 preparation, all MMP, BP, and control sera were routinely tested against native FN. FN (1 μg per well) was immobilized onto ELISA plates and was reacted with the tested sera by ELISA in the same manner as LN5.

LN5 dot blot analysis LN5 and FN (each at 2 μg per mL diluted in PBS) were immobilized onto nitrocellulose membranes using a dot blot chamber (Minifold, Schleicher & Schuell, Dassel, Germany). The membranes were blocked with milk buffer and were incubated overnight at 4°C with sera diluted at 1:200 in milk buffer. After four washes with TBS/0.15% Tween 20, the nitrocellulose membranes were incubated with anti-human IgG (Dako) for 1 h at RT. After repeated washes (4 × with TBS/0.15% Tween 20 and TBS), they were reacted with ECL (Amersham). Signal intensity was measured by LAS 1000 (Fuji Film, Düsseldorf, Germany). Reading of the tested sera was evaluated in comparison with the negative controls and the background reactivity to FN.

BP180 and BP230 ELISA All patients' sera were screened for the presence of IgG against BP180 and BP230, to verify the clinical diagnoses of MMP and BP, respectively. A more detailed description of the ELISA assays and statistical analyses used has been recently published (Hofmann *et al*, 2002; Thoma-Uszynski *et al*, 2004). In this study, we utilized an ELISA with a baculovirus-derived recombinant form of the BP180 ectodomain and two NH_2- and COOH-terminal recombinants of BP230 (Thoma-Uszynski *et al*, 2004).

Statistical analysis Statistical analysis was performed using the statistical software package SAS, version 8.2 (SAS Institute, Cary, North Carolina). ROC curves were compiled to evaluate the diagnostic properties of the LN5 ELISA with respect to correctly classified cases of BP and MMP (Witte, 2002). As there are no "natural" cutpoints for these ab levels, and as there are no previous studies that could have contributed evidence concerning an appropriate diagnostic threshold, an "optimum" cutpoint for the LN5 ELISA measurement values was determined data adaptively by choosing the OD values with the largest Youden index (YI = sensitivity + specificity–1). In detail: sequentially, each measurement observed in the group of patients (cases of BP and MMP, respectively) and controls, from the lowest to the highest value, was analyzed as a potential cutpoint. For each provisional cutpoint, the proportion of cases with values above that value that is correctly classified as BP of MMP, i.e. sensitivity, and the proportion of controls with values below that cutpoint that are correctly classified as healthy, i.e., specificity, is determined. Hence, the number of potential cutpoints corresponds to the number of case + controls, with increasing specificity and decreasing sensitivity accompanying increasing measurement values. From this list, the measurement with the maximum YI is chosen as a definite cutpoint. Several standard measures of diagnostic performance and a newly suggested summary measure, the "diagnostic odds ratio" (Glas *et al*, 2003), were calculated based on the cut-off point determined in the ROC analysis.

In view of the skewed distribution of OD values in BP and MMP patients, which was not amenable to transformation, OD values were analyzed with non-parametric statistical methods, e.g. Spearman' rank correlation coefficient and Wilcoxon–Mann–Whitney or Kruskal–Wallis test for overall equality of mean scores. The latter tests were performed using a Monte Carlo estimate of the exact p-values, supplemented with a 99% CI. In view of the exploratory role of statistical testing in our analysis, p-values were not adjusted for multiple testing; values less than 0.05 were considered significant.

The distribution of data is shown as a "box plot": the box shows first and thirsd quartile, the median (as line), and the arithmetic mean (as dot); the whiskers represent values below the first quartile and above the third quartile within the 1.5-fold inter-quartile range, respectively, and outliers beyond the whiskers are shown as squares.

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