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

Vassiliki Bekou,* Sybille Thoma-Uszynski,* Olaf Wendler,† Wolfgang Uter,‡ Susanne Schwietzke,* Thomas Hunziker,§ Christos C. Zouboulis,¶ Gerold Schuler,* Lydia Sorokin,|| and Michael Hertl*  

*Department of Dermatology, Friedrich Alexander University Erlangen-Nürnberg, Erlangen, Germany; †Interdisciplinary Center for Clinical Research (IZKF), Nikolaus Fiebig Center for Molecular Medicine, Friedrich Alexander University Erlangen-Nürnberg, Erlangen, Germany; ‡Department of Medical Informatics, Biometry and Epidemiology, Friedrich Alexander University Erlangen-Nürnberg, Erlangen, Germany; §Department of Dermatology, Campus Benjamin Franklin, Charité University Medicine, Berlin, Germany; ¶Department of Dermatology, University Hospital, Bern, Switzerland; ‡Department of Dermatology, Campus Benjamin Franklin, Charité University Medicine, Berlin, Germany; ||Department of Experimental Pathology, University of Lund, Lund, Sweden

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


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; Kirschig et al, 1995; Ghoresi et al, 1996), BP180, and the integrin [4 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 a6(4) 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-antibody directed against the extracellular domain of BP180 are related to the severity of BP (Dopp et al., 2000; Hofmann et al., 2002; Thoma-Uzynski et al., 2004). It is noteworthy that IgG auto-antibody 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., 1991; Burgeson 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 initially synthesized in a precursor form containing non-identical subunits of 200–165, 140, 155–105 kDa. The presence of LN5 in basement membranes is a characteristic feature of MMP (Nakatani et al., 1994; Burgeson 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 layer 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-antibody 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-antibody 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 1A) and immunoblot analysis (Fig 1B). 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 2A). Reactivity to LN5 was considered positive when the OD of the BP sera exceeded 0.230 units (Youden index of 0.285) (Fig 2B).

The diagnostic performance of each test was illustrated by receiver operating characteristic (ROC) curves (Fig 2A 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...
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 and 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 2C).

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.

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 2C). Statistically

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

<table>
<thead>
<tr>
<th></th>
<th>MMP (n = 24)</th>
<th>BP (n = 72)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutpoint</td>
<td>&gt;0.219</td>
<td>&gt;0.230</td>
</tr>
<tr>
<td>LN5 positive (n)</td>
<td>18</td>
<td>29</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>75.0% (53.3–90.2)</td>
<td>40.3% (28.9–52.5)</td>
</tr>
<tr>
<td>Specificity</td>
<td>84.3% (71.4–93.0)</td>
<td>88.2% (76.1–95.6)</td>
</tr>
<tr>
<td>Youden index</td>
<td>0.593</td>
<td>0.285</td>
</tr>
<tr>
<td>Diagnostic odds ratio</td>
<td>16.1 (4.9–53.2)</td>
<td>5.1 (1.9–13.4)</td>
</tr>
</tbody>
</table>

MMP, mucous membrane pemphigoid; BP, bullous pemphigoid; CI, confidence interval.

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 3A 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 3B. 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 a control protein, the clinical phenotype of the LN5-reactive MMP and BP patients is depicted.
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: 0.039–0.055 (Kruskal–Wallis test for overall homogeneity)) than patients with limited disease (Fig 5A). 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 5B). 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.

**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, conjunctivae, oral, and genital mucosa (Fig 6A). Serum samples taken over a period of 120 wk were tested for LN5-specific IgG by ELISA (Fig 6B) and by dot blot analysis (Fig 6C). LN5-specific IgG titers were maximal when extensive skin and mucous membrane involvement was present and slowly declined upon clinical improvement after administration of immunosuppressive 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 2/24 (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 7A and B). Remarkably, the sera from two MMP patients with extensive...
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; Hoshimoto et al., 1996; Lazarova and Yancey, 1996; Chan et al., 1997), whereas in the case of immunoblotting experiments

---

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

<table>
<thead>
<tr>
<th>Clinical involvement</th>
<th>MMP, n = 24</th>
<th>BP, n = 72</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Skin</td>
<td>Skin</td>
</tr>
<tr>
<td></td>
<td>extensive</td>
<td>limited</td>
</tr>
<tr>
<td>MMP</td>
<td>8 (33.3%)</td>
<td>10 (41.7%)</td>
</tr>
<tr>
<td>BP</td>
<td>50 (69.4%)</td>
<td>20 (27.8%)</td>
</tr>
</tbody>
</table>

---

"aOne MMP and two BP patients were in remission.

bExtensive disease, involvement of >15% of total body surface.

cLimited disease, involvement of <15% of total body surface.

---

**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).

**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.
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 immuno-

reactivity 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).

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 the 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 pathogenesis 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 pre-dominating 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 genitaloanal 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 °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, Colbe, 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% CO2. The cultures 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 on the second day to maintain optimal culture conditions.

For LN5 purification, conditioned media from 80% confluent 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-ethylmaleamide; 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 n3, 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 x 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-LN5 at 1:1000 (BM165, kindly provided by P. Rousselle), goat-anti-LN5 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 x 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 in a humidified environment at 5% CO2. The eluted protein fraction revealed the 200–165, 140, 155–105–80 kDa bands corresponding to the unprocessed and processed forms of the n3, 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).
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-Ușynski et al., 2004). In this study, we utilized an ELISA with a baculovirus-derived recombinant form of the BP180 ectodomain and two NH2- and COOH-terminal recombinants of BP230 (Thoma-Ușynski 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” cutoffs for these ab levels, and as there are no previous studies that could have contributed evidence concerning an appropriate diagnostic threshold, an “optimum” cutoff for the LN5 ELISA measurement values was determined data adaptively by choosing the OD values with the largest Youden index (Youden + sensitivity – specificity). 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 cutoff. For each provisional cutoff, 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 cutoff that are correctly classified as healthy, i.e., specificity, is determined. Hence, the number of potential cutoffs 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 Youden index is chosen as a definite cutoff. Several standard measures of diagnostic performance and a new, 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’s 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 third 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.

We are grateful to Dr Patricia Rousseille (Lyon, France) for the generous gift of mouse antibody BM165 and the SCC-25 cell line. We also thank Dr Leenstra, Dr Ru¨ckner-Tuderer (Freiburg, Germany) for the analysis of MMP sera against collagen VII, Dr Philippe Bernard (Reims, France), and Dr Nicolas Hunzelmann (Cologne, Germany) for providing sera of BP patients. This work was supported by grants from the European Community (QLG1-CT-2001-02007 to M. H.), the Interdisciplinary Center for Clinical Research IZKF (C9 to M. H.), and the ELAN founds (DE-Thoma- U0109111) of the University of Erlangen, Germany.

DOI: 10.1111/j.0022-202X.2005.23646.x

Manuscript received July 2, 2004; revised September 9, 2004; accepted for publication November 9, 2004.

Address correspondence to: Sybille Thoma-Ușynski, Department of Dermatology, Friedrich-Alexander-University of Erlangen-Nürnberg, Hartmannstraße 14, D-91052 Erlangen, Germany. Email: Sybille.Thoma-Ușynski@derma.med.uni-erlangen.de

References

Dopp R, Schmidt E, Chimanovitch I, Leverkus M, Brocker EB, Zillikens D: IgG4 and IgE are the major immunoglobulins targeting the NC16A domain of BP180 in Bullous pemphigoid. Serum levels of these immunoglobulins reflect disease activity. J Am Acad Dermatol 42:577–583, 2000
Ghohestani R, Kanitakis J, Nicolas JF, Cozzani E, Claudy A: Comparative sensitivities of indirect immunofluorescence to immunoblot assay for the de-
Rousseau P, Aumailley M: Kalinin is more efficient than laminin in promoting adhesion of primary keratinocytes and some other epithelial cells and has a different requirement for integrin receptors. J Cell Biol 125:205–214, 1994