Enzyme-linked immunosorbent assay using multimers of the 16th non-collagenous domain of the BP180 antigen for sensitive and specific detection of pemphigoid autoantibodies

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Abstract: Bullous pemphigoid (BP) and pemphigoid gestationis (PG) are acquired autoimmune subepidermal blistering diseases characterized by autoantibodies against the hemidesmosomal proteins BP180/type XVII collagen and BP230. In the vast majority of BP and PG patients, these autoantibodies bind to epitopes clustered within the 16th non-collagenous domain of BP180. An ELISA system for the detection of these autoantibodies was developed and evaluated using 16th non-collagenous domain (NC16A) tetramers instead of monomers. In contrast to antigens fused to large proteins used in the past for the detection of autoantibodies against type XVII collagen, tetrameric antigen fragments bearing a small hexahistidine tag allow for high expression levels without the need to cleave off the fusion partner. Using tetrameric BP180 NC16A, positive reactions were found in 106 (89.8%) of 118 randomly selected BP sera and in all of 20 (100%) randomly selected PG sera, whereas only 2.2% of a large cohort of control subjects were positive in this assay, including patients with rheumatoid arthritis (two of 107), progressive systemic sclerosis (two of 50), systemic lupus erythematosus (one of 72), and healthy blood donors (10 of 494). Thus, the sensitivity and specificity of the new anti-tetrameric NC16A ELISA were 89.9% and 97.8% respectively. Levels of circulating autoantibodies against BP180 paralleled disease activity in the pemphigoid patients. In conclusion, the use of tetrameric NC16A in ELISA results in a sensitive and specific tool for diagnosis and monitoring of BP and PG.

Key words: autoantigen – autoimmunity – BPAG2 – collagen – ELISA – tetramer

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Introduction

Bullous pemphigoid (BP) and pemphigoid gestationis (PG) are autoimmune subepidermal blistering diseases characterized by tissue-bound and circulating IgG autoantibodies against the dermal-epidermal junction (DEJ) (1,2). Autoantibodies in BP and PG are primarily directed against two hemidesmosomal proteins, bullous pemphigoid antigens 1 and 2, also referred to as the bullous pemphigoid antigens of 230 kDa (BP230) and 180 kDa (BP180) respectively (3–8). BP230 localizes to the intracellular hemidesmosomal plaque (9), whereas BP180 is a transmembrane protein with a type II orientation whose extracellular domain consists of 15 interrupted collagenous regions (Fig. 1a) (10,11). In addition to BP and PG, several other pemphigoid diseases are associated with autoantibodies against BP180, including linear IgA disease, mucous membrane pemphigoid and lichen planus pemphigoides (12).

The pathogenic relevance of autoantibodies against BP180 is supported by several lines of evidence: (i) the passive transfer of autoantibodies from mothers suffering from PG to the fetus induces transient skin blistering in
In vitro reactive with T and B cells of patients with BP and PG.

Lagenous domain (NC16A), harbours major epitopes domain of BP180, which is designated as the 16th non-collagenous domain; C15, 15th collagenous domain; NC16A, 16th non-collagenous A region.

Studies mapping the antigenic sites of human BP180 have shown that the extracellular membrane-proximal domain of BP180, which is designated as the 16th non-collagenous domain (NC16A), harbours major epitopes reactive with T and B cells of patients with BP and PG. Investigations using in vitro and in vivo models of BP suggested that the epitopes targeted by pathogenic autoantibodies also map to the NC16A region. Therefore, for the detection of autoantibodies against BP180, several ELISAs have been developed using recombinant forms of BP180, most commonly its NC16A domain. One of such test systems had been made commercially available. 

Small polypeptides like NC16A are also fused to larger partners with the intention to increase recombinant expression in the heterologous host. Instead of coupling to another protein, we fused four copies of the NC16A domain to a carboxyterminal polyhistidine-tag, which resulted in a protein of similar size as the GST fusion protein. This strategy allowed simple purification of the target protein under denaturing conditions with a high yield but without the need to cleave off the heterologous fusion partner. In addition to these technical advantages, the ELISA based on tetrameric NC16A proved to be a most sensitive and specific assay for the detection of autoantibodies against BP180.

Materials and methods

Human sera

Serum samples were obtained from patients with BP (n = 118), PG (n = 20), rheumatoid arthritis (n = 107), systemic scleroderma (n = 50), and systemic lupus erythematosus (n = 72) before initiation of treatment, as well as from healthy donors (n = 494). All pemphigoid patients were characterized by: (i) linear IgG and/or C3 deposition along the DEJ by immunofluorescence (IF) microscopy and (ii) circulating autoantibodies to the DEJ as revealed by IF microscopy. For the experiments conducted, we obtained institutional approval by the ethics committees at the Medical Faculties of the Universities of Lübeck and Würzburg (Institutional Board Projects 04–143, 06–090 and 37/98). In adherence to the Helsinki Principles, we obtained informed consent from all patients whose material was used in this study. To score disease activity, patients were seen weekly until lesions had cleared completely and then monthly for 1 year. On each visit, the number of blisters and erosions was recorded. More than 10 blisters/erosions corresponded to an activity score of 3, 1–10 blisters/erosions to a score of 2, and when no blister/erosion was detected, the score was 1. In addition, blood samples were taken for ELISA and indirect IF analysis on each visit.

Construction of recombinant vectors encoding for the tetrameric form of the NC16A domain of BP180

DNA sequence coding for the NC16A domain of human BP180 was cloned into a prokaryotic expression vector and expressed in E. coli following protocols described previously with modification. Briefly, DNA sequence data for human BP180 was retrieved from GenBank using the accesses...
sion number M91669 (10). cDNA fragments were obtained by polymerase chain reactions (PCR) on a chromosomal DNA purified from HEK 293 cells. Primers for PCR designed to contain appropriate restriction sites were synthesized by MWG Biotech (Ebersberg, Germany) (Table 1). The reaction was run in a thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA) for 30 cycles of 30 s denaturation at 94°C, 45 s annealing at 56°C, 30 s extension at 72°C, and finally 5 min extra extension for the last cycle. Resulting BP180 DNA fragments were digested with the appropriate restriction enzymes (Table 1) and subsequently cloned into linearized pET24d-N that had been digested with NcoI and Xhol. pET24d-N was generated by inserting the hybridized linker generated from oligonucleotides EP176 (CATGAGCCATCA TCATCATCATCATCATCATTCCATGGCGATATCCCTCG AGTAAG) and EP177 (TCGACTTACTCGAGGGATATC GCCATGAATGATGATGATGATGATGGCT) into pET24d (Novagen, Bad Soden, Germany) cut with NcoI and Xhol. Correct ligation and in-frame insertion of the DNA fragments were confirmed by DNA sequence analysis.

**Heterologous expression of recombinant forms of human BP180 in bacteria**

Recombinant fusion protein was expressed in *E. coli* RosettaBlue (DE3)pLacI (Novagen). For protein expression, a fresh bacterial colony was used to inoculate 10 ml LB medium supplemented with 50 and 34 μg/ml of kanamycine and chloramphenicol, respectively. The culture was incubated at 37°C for 12 h in a bacterial shaker at 130 rpm. The primary culture was used to inoculate 500 ml LB medium with appropriate antibiotics and incubated as described until the bacterial suspension reached an OD600 of 0.5–0.6. Protein expression was induced with 1 mM isopropyl β-d-thiogalactopyranoside for 3 h. Bacteria were harvested by centrifugation at 1800×g for 30 min and pellets were resuspended in 10 ml of 10 mM TRIS–HCl, 0.3 mM NaCl, 1 mM EDTA (pH 8.0). To release the recombinant proteins, the cell suspension was sonicated three times 20 s (Branson Sonifier, Branson Ultraschall, Dietzenbach, Germany) on ice immediately after the addition of Triton X-100 (Sigma-Aldrich, Taukirchen, Germany) to an end concentration of 1% (w/v). Lysed bacteria were centrifuged again at 15 000 × g for 20 min to sediment insoluble proteins. The supernatants were collected and used for purification of recombinant proteins. Pellets were solubilized in 10 ml 5 mM TRIS–HCl, 0.3 mM NaCl, 8 mM urea, 5 mM imidazol (pH 8.0) and tetrameric NC16A was purified by immobilized metal chelate affinity chromatography using Ni-NTA sepharose (Qiagen, Hilden, Germany) and 50 mM sodium acetate, 8 mM urea (pH 4.5) as eluent. Protein concentrations were determined by spectrophotometry at 280 nm (Eppendorf, Hamburg, Germany). Expression analysis was performed by SDS-PAGE and immunoblotting using a monoclonal antibody specific to hexahistidine (Merck Biosciences GmbH, Bad Soden, Germany). Immunofluorescence, SDS-PAGE and immunoblotting were performed as described (34,35).

**ELISA**

Microtiter plates (Nunc, Denmark) were coated with the tetrameric NC16A preparation (2.5 μg/ml in PBS, pH 7.5) overnight at 4°C, washed with PBS-0.05% (w/v) Tween-20 and blocked for 2 h with PBS-0.1% (w/v) casein. After washing, sera diluted 1:200 in PBS-0.1% (w/v) casein were added and allowed to react for 30 min. Bound antibodies were detected using anti-human IgG peroxidase conjugate and stained with tetramethylbenzidine (Euroimmun, Lubeck, Germany). All steps were carried out at room temperature. The optical density (OD) was read at 450 nm using an automated spectrophotometer (Spectra Mini, Tecan, Germany).

A highly positive index patient serum was used to generate a standard curve consisting of three calibrators. The cut-off for positivity was validated and optimized by receiver-operating characteristics (ROC) analysis as described.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Restriction sites</th>
<th>Primer sequences (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC16A-A</td>
<td>NcoI</td>
<td>F: ATACCATGGAGGAGGTGAGGAAGCTGAAGGCG</td>
</tr>
<tr>
<td></td>
<td>KpnI</td>
<td>R: TATGGTACCAATTGTCGCTCATTAGCCTC</td>
</tr>
<tr>
<td>NC16A-B</td>
<td>KpnI</td>
<td>F: ATAGGTACCACTCGAGGGATATC GCCATGAATGATGATGATGATGATGGCT</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>R: TATAAGGCTTATTTCCGTGTCCTCATTAAGCCTC</td>
</tr>
<tr>
<td>NC16A-C</td>
<td>HindII</td>
<td>F: ATAAAGGTCTAGGAGGTGAGGAAGCTGAAGGCG</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>R: TATGGATCATATTCCCTGTCCTCATTAAGCCTC</td>
</tr>
<tr>
<td>NC16A-D</td>
<td>BamHI</td>
<td>F: ATAGGTACCACTCGAGGGATATC GCCATGAATGATGATGATGATGGCT</td>
</tr>
<tr>
<td></td>
<td>XhoI</td>
<td>R: TATCTCGAGATTCTGTCCTCATTAAGCCTC</td>
</tr>
</tbody>
</table>

F, forward primer; R, reverse primer.

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Table 1. Primer sequences for polymerase chain reaction amplification of cDNA fragments of human BP180
below and was defined as 20 relative units (RU)/ml. The index sample was diluted in 1% (w/v) casein in PBS to yield those OD, which were defined as 2, 20 and 200 RU/ml. Relative units were calculated for all samples by this 3-point standard curve. The anti-BP180 ELISA based on monomeric recombinant NC16A expressed as a GST fusion protein was performed following the manufacturer’s instructions (MBL Co, Nagoya, Japan).

**Statistical analysis**

A ROC curve allows for exploring the relationship between the sensitivity and specificity of the tetramer ELISA for a variety of different cut-off points, thus allowing the determination of an optimal cut-off point for positivity. Therefore, to determine the cut-off value for the ELISA using tetrameric NC16A, we performed a ROC analysis by plotting on the X-axis the 1 – specificity (the false positive rate) and on the Y-axis the sensitivity (the true positive rate). Statistical analyses were performed using the EUROStat statistical package (Euroimmun). The Phi coefficient of association was computed using the VassarStats online (http://faculty.vassar.edu/lavy/tab2x2.html).

**Results**

**Preparation of the recombinant proteins**

The DNA sequence coding for human BP180 was cloned into a prokaryotic expression vector and expressed in *E. coli*. The protein, purified by metal chelate affinity chromatography, migrated consistent with its calculated mass of 36.6 kDa when separated by SDS-PAGE (Fig. 1b). A monoclonal antibody specific for hexahistidine recognized this recombinant form of NC16A by immunoblot analysis (Fig. 1c).

**Development of ELISA using tetrameric NC16A**

To determine the cut-off value of the newly developed immunoassay, we performed an ROC analysis of the ELISA readings with sera from 118 BP patients and 723 controls, including healthy donors (*n* = 494), patients with rheumatoid arthritis (*n* = 107), systemic scleroderma (*n* = 50) and systemic lupus erythematosus (*n* = 72). Based on a calculated clinical specificity of 98%, the cut-off was set at 20 RU/ml (Fig. 2).

**ELISA using tetrameric NC16A is a sensitive and specific tool to detect autoantibodies against BP180**

Using the cut-off defined by the ROC analysis, as shown in Fig. 3 106 (89.8%) of BP and 20 (100%) of PG sera showed positive scores, while two (1.9%) of 107 patients with rheumatoid arthritis, two (4%) of 50 patients with systemic scleroderma, and one (1.4%) of 72 systemic lupus erythematosus patients slightly exceeded the cut-off value (Table 2). Thus the sensitivity and specificity of the anti-tetrameric NC16A ELISA were 89.8% (106/118) and 97.8% (224/229) respectively. In addition, 10 (2%) of 494 healthy donors showed positive scores using anti-tetrameric NC16A ELISA. Sera from BP patients and controls were tested in parallel using the commercially available anti-BP180 ELISA (MBL). By this immunoassay, in addition to
ELISA using tetrameric NC16A is useful for monitoring levels of autoantibodies against BP180 in BP patients

To monitor quantitatively serum levels of autoantibodies to BP180 during the course of the disease, in four BP patients, reactivity with the tetrameric NC16A domain was assayed by ELISA. Serum levels of autoantibodies were shown to parallel disease activity in these BP patients (Fig. 4).

Discussion

ELISA systems for the detection of autoantibodies against the bacterially expressed immunodominant NC16A domain of BP180 have already been in use and proved to be sensitive and specific tools for diagnosing pemphigoid diseases. These ELISAs based on recombinant NC16A fused to GST to increase the low expression rate of the small NC16A polypeptide in a bacterial host. The fusion to GST, however, resulted in the necessity to either cleave off the GST tag from GST-NC16A before application in the ELISA system or conducting two ELISA tests in parallel one using the fusion protein as antigenic substrate, the other using GST alone. Subsequently, the difference between the two OD readings was determined to measure specific autoantibody levels (30). In the present study, to overcome these limitations, a tetrameric NC16A fused to a small polyhistidine-tag was expressed, purified to homogeneity and used to establish an ELISA. Due to the tetramerization of NC16A, the size of the recombinant peptide is in the optimal range for bacterial expression, which results in a high expression level.

ELISA tests using large domains of desmogleins have been established for pemphigus diseases, in which pathogenic autoantibodies may target conformational or non-conformational epitopes (36,37). In BP, autoantibodies may react with epitopes outside the NC16A domain of BP180 (38). Therefore, recombinant forms of full-length and of the entire extracellular domain BP180 have been generated and used to develop immunoassays (31,39,40). However, the expression of collagenous proteins of high molecular mass requires eukaryotic expression systems and the yield of these recombinant forms is relatively low. In addition, no or low improvement in sensitivity and specificity over ELISAs using recombinant BP180 NC16A expressed in bacteria was documented using these assays (31,39,40). Indeed, although longer recombinant forms of BP180 are available in several laboratories, so far no ELISA using these proteins has been developed for commercial use. We and others therefore concluded that recombinant forms of the immunodominant domain of BP180 are the most appropriate for developing an immunoassay to detect autoantibodies in BP and PG patients (16,17,23,30).

<table>
<thead>
<tr>
<th>Sera</th>
<th>Number</th>
<th>Tetrameric NC16A ELISA</th>
<th>GST-NC16A ELISA (MBL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>118</td>
<td>106 (89.8)</td>
<td>105 (89.0)</td>
</tr>
<tr>
<td>PG</td>
<td>20</td>
<td>20 (100)</td>
<td>20 (100)</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>107</td>
<td>2 (1.9)</td>
<td>5 (4.7)</td>
</tr>
<tr>
<td>Systemic sclerosis</td>
<td>50</td>
<td>2 (4.0)</td>
<td>4 (8.0)</td>
</tr>
<tr>
<td>Systemic lupus erythematosus</td>
<td>72</td>
<td>1 (1.4)</td>
<td>3 (4.3)</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>118</td>
<td>89.8%</td>
<td>89.0%</td>
</tr>
<tr>
<td>Specificity</td>
<td>229</td>
<td>97.8%</td>
<td>94.8%</td>
</tr>
</tbody>
</table>

Values in parentheses are indicated as percentage.
BP, bullous pemphigoid; PG, pemphigoid gestationis.

*Table 2. Sensitivity and specificity of the anti-tetrameric NC16A ELISA in comparison with the commercially available anti-BP180 ELISA test (MBL)*

Detection of autoantibodies using tetramer ELISA correlates with findings by indirect immunofluorescence microscopy in BP patients

Findings by ELISA using tetrameric BP180 NC16A and indirect IF microscopy were evaluated in 118 BP patients and are summarized in Table 3. Positive findings by both indirect IF microscopy and ELISA highly correlated as revealed by the calculated Phi coefficient of association (0.72). Discrepant results were observed in 17 (14.4%) BP patients, including positive ELISA with negative IF in six (5%) patients and negative ELISA with positive IF in 11 (8.5%) patients.

*Table 3. Correlation of findings by indirect immunofluorescence (IIF) and ELISA for the detection of autoantibodies*

<table>
<thead>
<tr>
<th></th>
<th>IIF positive</th>
<th>IIF negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA positive</td>
<td>100</td>
<td>6</td>
</tr>
<tr>
<td>ELISA negative</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

†Tetramer BP180 NC16A ELISA.
When used to assess serum levels of antibodies to BP180, the new anti-tetrameric NC16A ELISA showed a sensitivity of 89.8% and 100% in BP and PG patients respectively. In the same group of patients and controls, the commercially available anti-BP180 ELISA based on NC16A fused to GST showed a similar sensitivity. Importantly, however, the new anti-tetrameric NC16A ELISA demonstrated a slightly higher specificity compared with the conventional test system (97.8 vs 94.8%). In addition, findings obtained using the newly developed assay correlated with those by indirect IF microscopy. However, the ELISA using tetrameric BP180 NC16A is detecting autoantibodies dubbed to be pathogenic (specific to BP180) and was shown to correlate with disease activity. In contrast, indirect IF microscopy does not differentiate between autoantibodies with different molecular specificity, which also probably exert different pathogenic effects.

Tetramerization of antigen may have additional advantages when compared with antigen monomers fused to large protein tags, including enhancing the binding of peptides or proteins to antigen-specific T cells and autoantibodies. Multimerization of the MHC/peptide complexes increases the avidity of their interaction with T-cell receptors and enables the complexes to be used for detection of antigen-specific T cells (41). Multimers of epitopes have been shown to increase the immunogenicity of antigens and increase the immune response when used for immunization (42). Recently, tetramerization of self-antigen has been shown to increase its immunoreactivity and to allow discrimination of autoantibodies to native and denatured protein (43). Probably, multimerization of NC16A may also have increased its immunoreactivity in our ELISA system and thus contributed to the high sensitivity and specificity of this assay.

In BP and PG, different studies demonstrated that serum levels of autoantibodies to BP180, as measured by ELISA, are in parallel to the disease activity (15–17). In line with these observations, we found levels of autoantibodies measured by the anti-tetrameric NC16A ELISA to reflect closely also disease activity in BP patients. Thus, the ELISA using the tetrameric NC16A domain is not only a helpful tool to diagnose the disease, but also suitable to guide treatment decisions, especially in those patients who are free from skin lesions and yet receive treatment.

In conclusion, to increase the immunoreactivity of the BP180 antigen, a new molecular approach was devised using recombinant antigen multimers. A tetramer of the immunodominant NC16A domain of BP180 was generated and an ELISA system developed. This provides a sensitive and most specific test for detection of circulating autoantibodies against BP180 in BP and PG. Further studies should assess the usefulness of the anti-tetrameric NC16A ELISA in other pemphigoid diseases, including mucous membrane pemphigoid and lichen planus pemphigoides.

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**References**


