

histopathological evidence of subepidermal blister formation; 3) linear IgG and/or C3 deposits at the dermoepidermal junction of perilesional skin by direct immunofluorescence; and 4) IgG reactivity with the epidermal side of human saline split skin or IgG reactivity with BP180 or BP230.

Severity of BP was classified according to the extent and number of blisters as either limited disease (only few bullae at limited areas of the body involving up to 20% of total body surface) or extensive disease with total body surface involvement greater than 20% and wide-spread bullae.

2.2 Production of recombinant BP180 ectodomain

The ecto-subdomains of BP180 were produced by stable expression in human embryonic kidney 293-EBNA cells (Invitrogen) as reported (Areida et al., 2001; Nishie et al., 2010). Briefly, the recombinant protein encoding for the subdomains of human BP180 ectodomain (BP180-A, B, C and D) were concentrated by ultrafiltration and purified by an anion exchange column and Superose 6 column. Fractions containing recombinant fragments of BP180 (A, B, C and D) were detected by SDS gel electrophoresis and Western blotting.

2.3 Electrophoresis and Western Blot Analysis

SDS gel electrophoresis (Laemmli, 1970) and Western blot analysis (Giudice et al., 1993) were performed according to standard procedures. 6 % polyacrylamide gels were used for rColXVII-A and -B, and 10 % polyacrylamide gels were used for rColXVII-C and -D. Polyclonal rabbit antiserum NC16a was used 1:1000 diluted for rColXVII-A, -B, and -C (Schumann et al., 2000) and polyclonal chicken antiserum Col17ecto-1 was used 1:20 diluted for rColXVII-D. The antisera were a generous gift of Dr. L. Bruckner-Tuderman. Goat-anti rabbit (1:1000; Dako) or goat antichick (1:100; Dako) antibodies conjugated to horseradish peroxidase were used as secondary antibodies, respectively.

2.4 ELISA

The ELISA using BP180 NC16A as the target antigen was performed as described previously (Schmidt et al., 2000). Briefly, wells were coated with purified forms of recombinant BP180-A, B, C and D respectively. Subsequently, wells were incubated with patient and control serum samples, and bound antibodies were visualized by peroxidase-conjugated rabbit antihuman IgG (DAKO).

For detection of IgA autoantibodies, 40-well microtiter plates were coated with 1.4 µg/ well of recombinant BP180 ectodomains in 0.1 M bicarbonate buffer (pH 9.6), overnight at 4 °C. After washing with 0.05% Tween20-PBS (w/v) and subsequent 1 h blocking with 2% BSA-PBS (w/v) the plates were incubated for 1 h with 1:50 diluted sera in 1% BSA-0.05% Tween20-PBS (w/v). Bound antibodies were detected using a 1000-fold dilution of a horseradish-peroxidase (HRP) conjugated rabbit anti-human IgA antibody (ab8510, Abcam) and orthophenylene diamine (Dako). All steps were carried out at room temperature. The optical density (OD) was read at 490 nm using an automated spectrophotometer

(Sirius HT-TRF, MWG). Each serum was tested in duplicate. The cut-off for positivity was validated and optimized by receiver-operating characteristics (ROC) analysis as described below and was defined as 0.48 OD units at 490 nm.

3. Results

3.1 Production Recombinant BP180 Subdomains:

To investigate the reactivity of IgA autoantibodies from sera of bullous pemphigoid patients, we have produced several recombinant subdomains of the BP180 ectodomain in mammalian 293 cells. These subdomains included the full length ectodomain BP180-A (pos.527-1497), a C-terminal truncated form thereof BP180-B (pos. 527-1482), the largest collagenous BP180-C (pos. 527-808), and a short C-terminal subdomain BP180-D (pos. 1188-1497). These recombinant fragments are schematically summarized in [Fig-1]. After ion exchange chromatography followed by gel filtration chromatography, the highly purified recombinant subdomains with apparent molecular masses of 101 kDa (BP180-A), 96 kDa (BP180-B), 30 kDa (BP180-C), and 33 kDa (BP180-D) which corresponded well with the calculated values of collagenXVII [Fig-2], when separated by SDS-PAGE.

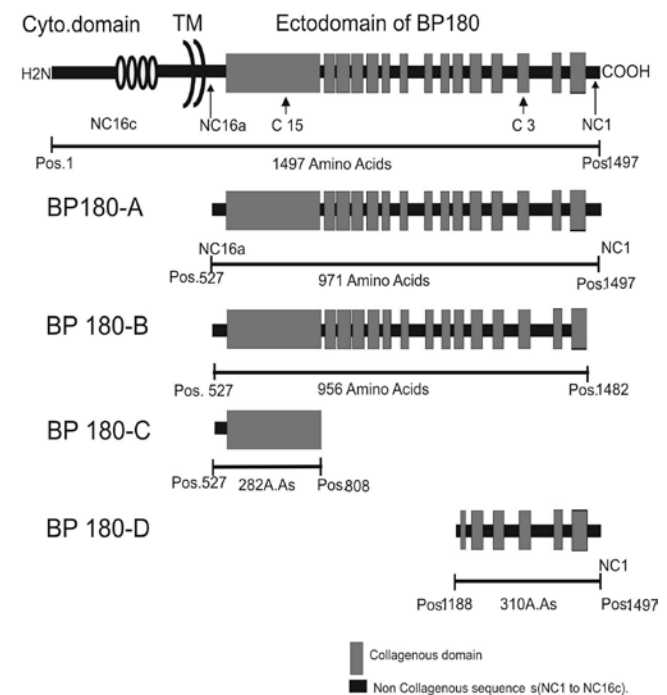


Fig-1: Schematic representation of recombinant ectodomains of collagen XVII (BP 180) used in this study: The extracellular domain consists of a series of 15 collagenous domains (C 1 through C 15, solid vertical boxes) and stretches of non collagenous domains (NC 1 through NC 16a, horizontal bars). The BP180-A recombinant extends from amino acid 527 to 1497. BP180-B recombinant extends from amino acid 527 to 1482. BP180-C recombinant extends from amino acid 527 to 808. BP180-D recombinant

extends from amino acid positions 1188 to 1497. The total numbers of amino acid residues for each of the constructs are indicated. GH; globular head. TM; transmembrane region.

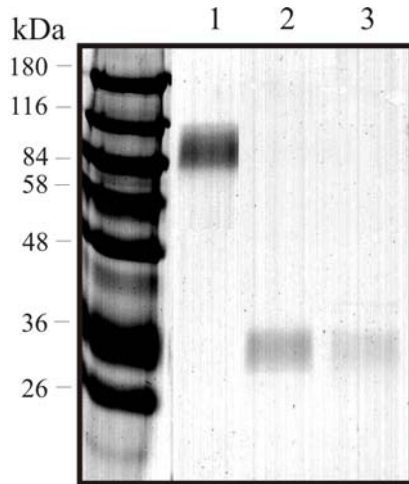


Figure 2: Purification of recombinant BP180 proteins. The purified recombinant BP180 proteins separated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie Blue. The BP180-A, C and D migrates around 100 kDa (lane 1), 30 kDa (lane 2), and 33 kDa (lane 3) respectively.

3.2 Immunoblotting of recombinant BP180 subdomains with IgA bullous pemphigoid patients' sera

IgA autoantibodies from reference IgA bullous pemphigoid patients' sera (n = 30) recognized the recombinant BP180 ectodomain by immunoblotting [Fig-3]. Immunoblotting of BP serum samples was reacted with different recombinant fragments of BP180, NC16A (BP180-A, B and C) and recombinant fragments of BP180, NC1 with C-terminal (BP180-A and D) as shown in figure-3 [lane 1, 2 and 3] and [lane 4, 5 and 6] respectively. None of the normal human sera reacted with the autoantigens and IgA antibodies from none of the tested sera bound to the precipitated proteins of the empty vector transfected EBNA 293 cell culture medium [Fig-3, lane 7].

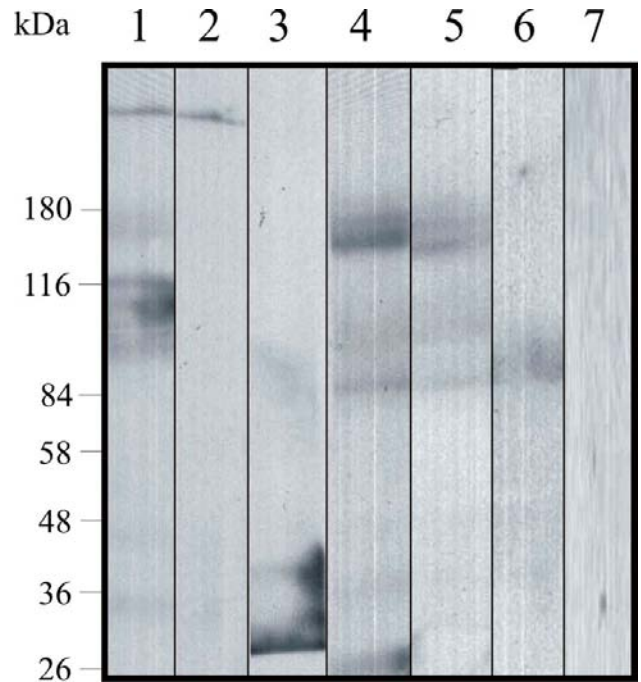


Figure 3: Immunoblotting of recombinant BP180 subdomains with IgA bullous pemphigoid patients' sera: Recombinant BP180-A (lanes 1, 4 and 5) recombinant BP180-B (lanes 2) recombinant BP180-C (lanes 3) recombinant BP180-D (lanes 6) and control using empty vector (lane 7).

3.3 ELISA analysis using BP180 recombinant proteins

The results of the ELISA were correlated with those of immunoblotting. Serum from BP patients showed IgA reactivity with the recombinant BP180 ecto-subdomain. Therefore, a sensitivity and a specificity of 86. % with BP180 (A and C) and 73% with BP180-D, respectively, were calculated for the ELISA detecting IgA autoantibodies against BP180 in patients with IgA pemphigoid [Table-1].

Table 1: ELISA analysis using BP180 recombinant proteins
 Total number of tested sera = 30

| | Indirect immunofluorescence | Direct immunofluorescence | Immunoblot with recombinant BP180-A-ectodomain | Immunoblot with recombinant BP180-C-ectodomain | Immunoblot with recombinant BP180-D-ectodomain |
|----------------------|-----------------------------|---------------------------|--|--|--|
| ELISA BP180 positive | 25 (83%) | 20 (66%) | 24 (80%) | 26 (86%) | 22 (73%) |
| ELISA BP180 negative | 5 (17%) | 10 (34%) | 6 (20%) | 4 (14%) | 8 (27%) |

4. Discussion

In the present study, we characterized the autoimmune response in 30 patients with the BP autoantibodies against recombinant BP180. IgG and IgE, IgA autoantibodies are present to different extent in pemphigoid diseases (Kromminga et al., 2000). While pemphigoid patients showing a predominant IgA autoimmune response directed against BP180 are diagnosed as linear IgA disease, IgA autoantibodies may complement a dominant IgG response in bullous pemphigoid or mucous membrane pemphigoid (Yancey et al., 1999; Schmidt et al., 2000; Mihai et al., 2007). However, quantitative assays for measuring the levels of serum IgA autoantibodies in pemphigoid diseases have not been established yet. Therefore, in the present study, we characterized ELISA and immunoreactivity using recombinant BP180 for the detection of IgA autoantibodies.

For detecting the IgA autoantibodies in pemphigoid diseases we have used a recently generated recombinant form of the ecto- subdomain of BP180 (Areida et al., 2001). The ecto-subdomain of the autoantigen was expressed in a human embryonic kidney 293 cell lines to ensure optimal posttranslational modifications of the protein, which were shown to influence the binding of pemphigoid autoantibodies of the IgG class (Zimina et al., 2008). The expression of the antigen in mammalian cells and the fact that our recombinant forms of BP180 contains its entire ectodomain significantly raised the sensitivity and specificity of the immunoassay and strongly support its use for the detection of IgA autoantibodies for the diagnosis of pemphigoid diseases.

The ELISA showed highly sensitive and specific for the detection of IgA autoantibodies in bullous pemphigoid. Since IgA reactivity with the epidermal side of the salt-split human skin by indirect IF microscopy was an inclusion criterion of the study patients. The observation that IgA autoantibodies against BP180 were detected in bullous pemphigoid patients is in line with previous findings (Schmidt et al., 2000, Kromminga et al., 2000). Our finding, that a significant proportion of the bullous pemphigoid patients show IgA autoantibodies against the ectodomain of BP180 suggests that the newly developed IgA ELISA may be a useful ancillary diagnostic tool in patients with bullous pemphigoid.

In addition to facilitating translational research focusing on the IgA autoimmune response in pemphigoid diseases, the ELISA could complement or replace the traditional semi-quantitative, observer-dependent and time-consuming IF microscopy on salt-split skin and immunoblotting using concentrated conditioned supernatant of cultured keratinocytes. In contrast to IgG reactivity (Di Zenzo et al., 2008), the ELISA levels of BP180-specific IgA correlated well with the IgA reactivity by indirect IF microscopy. In a recent study, we have investigated the potential of neoepitope-specific rabbit IgG antibodies to induce dermal-epidermal separation in an *ex vivo* assay (Nishie et al., 2010). Using developed ELISA with further recombinant forms of the BP180 ectodomain, which may better reproduce the neoepitopes of the native shed ectodomain, may facilitate addressing the diagnostic relevance of

measuring specifically the neoepitope-specific IgA autoantibodies.

We further generated a recombinant protein encompassing the extracellular domain of BP180 and found reactivity against this protein in a third of our CP sera. Previous studies demonstrated that the intracellular domain of BP180 mediates interaction between BP180 and the $\beta 4$ subunit of $\alpha 6\beta 4$ integrin, which is important for the stabilization of the DEJ (Liu et al., 1995). Interestingly, all of our sera reactive with the extracellular portion of BP180 recognized additional epitopes on the BP180 ectodomain. Initially, autoantibodies against extracellular epitopes may mediate blister formation as suggested by the experimental mouse model of BP where antibodies to the murine homologue of the human BP180 NC16A domain cause dermal-epidermal separation (Stanley et al., 1981). One may speculate, however, that during the later course of the disease, after some injury to the basal keratinocyte has occurred, extracellular epitopes may also become accessible for the immune response.

5. Conclusion

We demonstrated that autoantibodies in patients with BP reacted with distinct epitopes clustered within the N-terminus of BP180 NC16A and C-terminus NC1. Our findings suggest that ELISA and immunoblotting using the NC16A and C-terminus NC1 domains as the target antigen are suitable tools for the diagnosis of BP and for monitoring disease activity during treatment.

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