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The majority of bullous pemphigoid and herpes gestationis serum samples react with the NC16a domain of the 180-kDa bullous pemphigoid antigen

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Abstract A series of previous studies have indicated that the NC16a domain of the 180-kDa bullous pemphigoid antigen (BP180) is the most immunogenic and probably pathogenic region in both bullous pemphigoid and herpes gestationis. To confirm these previous results by a large-scale study, we examined serum from 154 bullous pemphigoid and 43 herpes gestationis patients using an immunoblot technique with the BP180 NC16a domain fusion protein, which had been prepared using cDNA obtained from a human keratinocyte cDNA library by polymerase chain reaction amplification. This fusion protein was recognized by 90% of bullous pemphigoid and 79% of herpes gestationis serum samples, but not by any other disease or normal control serum samples. These results indicate that this system is very sensitive and specific for the detection of anti-BP180 antibodies and should be useful for the diagnosis of various subepidermal bullous diseases.

Key words Autoantigen · Basement membrane zone · Bullous pemphigoid · Immunoblot · Recombinant protein

Introduction

Anti-basement membrane zone (BMZ) antibodies in serum from bullous pemphigoid (BP) patients react with the 230-kDa BP antigen (BP230) and the 180 kDa BP antigen (BP180) [1–5], and those in serum from herpes gestationis (HG) patients recognize predominantly the BP180 [6]. cDNA cloning for BP230 has revealed that it is a protein with a structure similar to desmoplakin [7, 8].

Because autoantibodies are considered to be inaccessible to intracellular molecules, the role of the anti-BP230 antibodies in the pathogenesis of BP is currently unknown. cDNA cloning for BP180 has shown that it is a transmembranous protein of type 2 orientation, with an extracellular domain consisting of 15 collagen-like domains [9, 10]. Recent study using recombinant proteins has indicated that autoantibodies in serum from most BP and HG patients recognize the NC16a domain of BP180, which comprises the 76 amino acid stretch positioned between the membrane spanning domain and the first extracellular collagenous domain [11, 12]. Furthermore, using a mouse animal model, Liu et al. have recently shown that the antibodies to BP180 play a crucial role in blister formation [13].

In this study, to further evaluate the importance of the NC16a domain in antigenicity of BP180, we analysed the reactivity of a large collection of BP and HG serum samples with a bacterial fusion protein of the NC16a domain.

Materials and methods

Serum samples

Serum samples were obtained from 154 BP patients, 43 HG patients, 10 each of pemphigus vulgaris and pemphigus foliaceus patients, and 30 normal volunteers. Diagnosis of BP and HG was made by their typical clinical, histopathological and immunopathological features. The circulating anti-BMZ antibodies were detected in all the BP samples at a titre of 1:40 to 1:5120 by immunofluorescence of normal human skin sections. In contrast, only 24 (56%) of the 43 HG serum samples showed anti-BMZ antibodies at a titre of 1:10 to 1:40, except for one case at a titre of 1:160; the remaining 19 HG samples did not show the antibodies. All samples were kept at -80°C for long-term storage and at 4°C in the presence of 0.1% NaN₃ for less than 1 month during the experiments.

Immunoblot analysis

Immunoblotting of extracts of EDTA-separated normal human epidermis was performed as described previously [4].

We prepared bacterial fusion protein containing the NC16a domain of BP180. A human keratinocyte cDNA library was prepared

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as reported previously [14]. In order to obtain cDNA covering the entire NC16a domain, we prepared a forward primer (5'-caaggatc-cGAGGAGGTGAGGAAGCTG-3'; capital letters indicating nucleotides 1573–1590) and a reverse primer (5'-ccgaatTCGGAGA-TTTCATTTTCCTG-3'; capital letters indicating nucleotides 1782–1803), according to sequence data reported previously by Giudice et al. [10]. EcoRI and BamHI sites (shown by lower case letters) were incorporated to facilitate subsequent subcloning. cDNA was amplified by the polymerase chain reaction using the human keratinocyte cDNA library as template (94°C 1 min, 55°C 1 min, 72°C 1 min; 28 cycles), purified with a PCR purification kit (QIAGEN, Chatsworth, Calif., USA), and digested with BamHI and EcoRI. Gel-purified cDNA was then subcloned into the bacterial expression vector pGEX-2T (Pharmacia, Uppsala, Sweden) which had been digested with EcoRI and BamHI and gel purified. The fusion protein with glutathione-S-transferase was induced by the addition of isopropyl- β -thiogalactopyranoside, and purified on a glutathione sepharose 4B column (Pharmacia), according to the manufacturer's recommendations. Glutathione-S-transferase was also prepared by the same method using the original pGEX-2T vector.

Immunoblotting using this fusion protein as antigen source was performed, mainly as previously reported [4]. Briefly, purified fusion protein was resolved in Laemmli's sample buffer, and applied to 15% SDS polyacrylamide gel. After transfer to a nitrocellulose membrane, the blots were reacted successively with 1:40 diluted serum and 1:100 diluted peroxidase-conjugated antihuman IgG antiserum (DAKO, Copenhagen, Denmark), and colour was developed using 4-chloro-1-naphthol.

Results

With immunoblot analysis of normal human epidermal extracts, 110 (71%) and 91 (59%) of the 154 BP serum samples reacted with BP230 and BP180, respectively, and 11 (26%) and 22 (51%) of the 43 HG serum samples reacted with BP230 and BP180, respectively (Table 1). None of the pemphigus or normal control serum samples reacted with these antigens, although pemphigus serum reacted with either pemphigus vulgaris or pemphigus foliaceus antigen.

We verified that we had obtained the correct BP180 NC16a domain fusion protein by confirming that the sizes of the PCR-amplified cDNA (about 250 bp) and the fusion protein (about 35 kDa, including 26 kDa of glutathione-S-transferase) were as expected. With immunoblot analysis of BP180 NC16a domain fusion protein, 138 (90%) of the 154 BP serum samples and 34 (79%) of 43 HG samples reacted with this fusion protein (Table 1, Fig. 1). All the BP and HG samples showed reactivity with the whole BP180 molecule in epidermal extracts re-

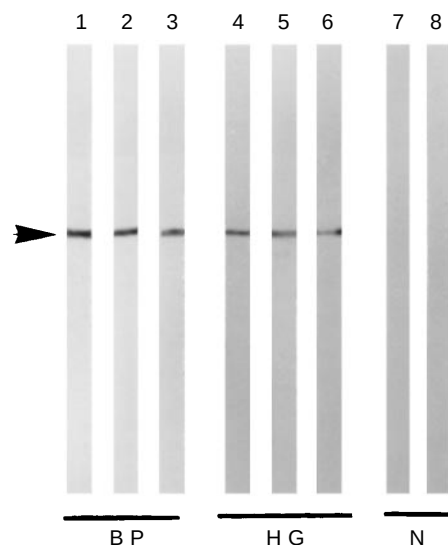


Fig. 1 Serum from the majority of BP and HG patients reacted with the fusion protein of the BP180 NC16a domain. BP serum (lanes 1–3) and HG serum (lanes 4–6), but not normal serum (lanes 7, 8), reacted with the fusion protein (arrow)

acted with the fusion protein, except for one case. It should be emphasized that 10 HG serum samples which did not react with the BMZ by indirect immunofluorescence did react with this fusion protein. None of the samples used in this study reacted with the glutathione-S-transferase.

Discussion

In this study we examined the reactivity of BP and HG serum with a bacterial fusion protein covering the BP180 NC16a domain using an immunoblot technique, and showed that more serum samples from both BP and HG recognized the fusion protein than BP180 in epidermal extracts. These results confirm previous findings [11, 12] that serum from the majority of BP and HG patients recognizes a common immunodominant region on BP180, and also indicate that the fusion protein is more suitable than epidermal extracts as an antigen source for detecting circulating autoantibodies against BP180 in BP and HG.

Compared with BP serum, HG serum recognized the fusion protein less frequently. This is probably due to a very low titre of anti-BMZ antibodies in HG serum. Anti-BMZ antibodies were detected in only 56% of the HG serum samples, whereas 79% of the HG serum samples detected the BP180 fusion protein by immunoblotting, indicating that this immunoblot system is very useful for the diagnosis of HG.

The reason why more serum samples recognized the fusion protein representing only a part of the BP180 molecule than recognized the whole BP180 molecule in the epidermal extracts is unclear. An explanation could be different amounts of antigens in the two antigen sources, i.e. more protein is present in the fusion protein sample than in human epidermal extracts. Alternatively, the anti-

Table 1 Summary of immunoblot analysis of human epidermal extracts and fusion protein of the BP180 NC16a domain

Disease	Number of serum samples	Epidermal extracts		BP180 NC16a fusion protein
		BP230	BP180	
BP	154	110 (71%)	91 (59%)	138 (90%)
HG	43	11 (26%)	22 (51%)	34 (79%)
PV	10	0	0	0
PF	10	0	0	0
Normal	30	0	0	0

bodies could access the epitopes on the recombinant protein more easily than those on the whole molecule.

One BP serum sample reacted with BP180 in the epidermal extracts but not with the fusion protein, suggesting that the epitope for this serum may not be present on the NC16a domain.

The percentages of BP and HG serum samples that reacted with the fusion protein were in general higher than those reported previously [11]. This difference may be explained by the fact that, while our fusion protein covered the entire NC16a domain, the previous study used fusion proteins containing parts of this domain, and in particular lacking the first 17 amino acids [11].

BP180 used to be considered a relatively minor BP antigen and was detected less frequently by BP serum than BP230 with conventional immunoblotting of epidermal extracts ([3, 4]; and in this study). However, in this study, by using fusion protein of the NC16a domain we were able to improve the sensitivity of the assay for detection of anti-BP180 autoantibodies and showed that serum from most BP and HG patients contained anti-BP180 antibodies. Considering that the antibodies against the NC16a domain play a crucial role in blister formation [13], the results of this study suggest that serum obtained from essentially all BP or HG patients with active disease should contain antibodies to this region of BP180. Furthermore, the molecular probe used in this study should facilitate future investigation and diagnosis of BP and HG.

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