# International multicenter study of the humoral autoimmune response in patients with bullous pemphigoid and mucous membrane pemphigoid: antigenic characterization and fine epitope mapping study

**M. Elisabet Parera Amer*, Noèlia Armíger Borràs*, Pilar Iranzo¶, Cassian Sitaru§, Ramon Quintana±, Giovanni Di Zenzo#, Giovanna Zambruno#, Ramon M. Pujol Vallverdú*, Josep E. Herrero González***

*Department of Dermatology. Hospital del Mar, Institut Municipal d'Investigació Mèdica, Parc de Recerca Biomèdica, Universitat Autònoma de Barcelona, Barcelona. Spain. ¶Department of Dermatology, Hospital Clínic Provincial. Barcelona. Spain. §Department of Dermatology, Uniklinik Freiburg. Germany. ±Department of Ophthalmology, Hospital de la Maternitat, Corporació Sanitària Clínic. Barcelona. Spain. #Istituto Dermopatico dell'Immacolata, IDI-IRCCS, Rome, Italy

## Background

Bullous pemphigoid (BP) and mucous membrane pemphigoid (MMP) are subepidermal blistering diseases associated with autoimmunity to type XVII collagen. Several epitope mapping studies have demonstrated epitope clustering in diverse regions of its ectodomain, mainly the membrane-proximal non-collagenous 16A domain (NC16A). Additionally, BP and MMP patients may show a lower reactivity to other extracellular and intracellular epitopes of type XVII collagen[1-4]. The pathogenic role of antibodies against BP180 has been demonstrated in *in vitro* and *in vivo* models of the disease[5-8]. However, the pathogenic relevance of specific autoantibodies to regions other than NC16A remains to be elucidated.

## Aims

To characterize the antigenic specificity of autoantibodies against epidermal basement membrane proteins and to analyze the fine specificity of anti-type XVII collagen antibodies of BP and MMP sera.

## Material and methods

Sera from 19 BP and 20 MMP patients were collected. The diagnosis was confirmed by clinical, histopathological and direct immunofluorescence findings. Humoral autoimmune response against basement membrane proteins was analyzed by indirect immunofluorescence on salt-split skin and by immunoblot with epidermal, keratinocyte and amniotic extracts. Human epidermal and amniotic extracts were prepared according to previous studies[8,9].

9 recombinant fragments of the BP180 ectodomain were also obtained as described elsewhere[1,10]. These fragments are shown in **Figure 1.** IgG reactivity against these GST-fusion proteins was determined by immunoblot and ELISA.

Finally, the blister-inducing potential of the sera was analyzed *in vitro* using cryosections of human healthy skin as a substrate. The extension of the dermal-epidermal separation was determined by two different investigators.

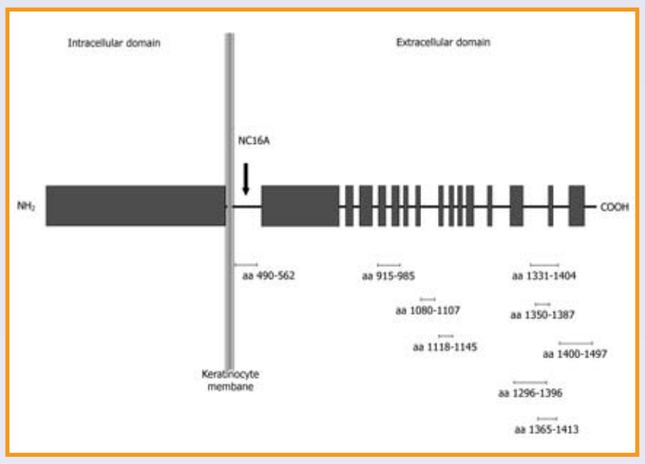


**Figure 1. Type XVII collagen schematic diagram. Recombinant fragments used are depicted.**

## Results

Indirect immunofluorescence revealed circulanting IgG antibodies binding to the epidermal side of salt-split skin in 84% BP and 15% MMP cases. IgA antibodies were detected in 68% BP and 20% MMP patients (**Figure 2**). IgG binding to the dermal side of salt-split skin was detected in only one MMP patient.

Immunoblot assay results are shown in **Table 1**. BP180 was detected in 36-55% BP and <5% MMP patients depending on the used extract. BP230 was detected in 26-72% BP and 0-21% MMP patients. LAD1 was detected in 5.5-21% BP and 0-26% MMP patients. LABD97 was detected in 0-21% BP and <5% of MMP patients. The best immunoblotting results were obtained using epidermal and keratinocyte extracts. IgG reactivity against recombinant fragments of BP180 analyzed by immunoblot and ELISA techniques are shown in **Table 2**. Serum autoantibodies against the NC16A domain and a C-terminal fragment spanning residues 1296-1413 were detected by ELISA in 74% and 47% of BP, and 35% and 65% of MMP patients, respectively. Induction of *in vitro* dermal-epidermal separation was observed in 79% of BP patients and 15% of MMP sera (an example is shown in **Figure 3**). The extent of in vitro induced dermal-epidermal separation is shown in **Table 3**.

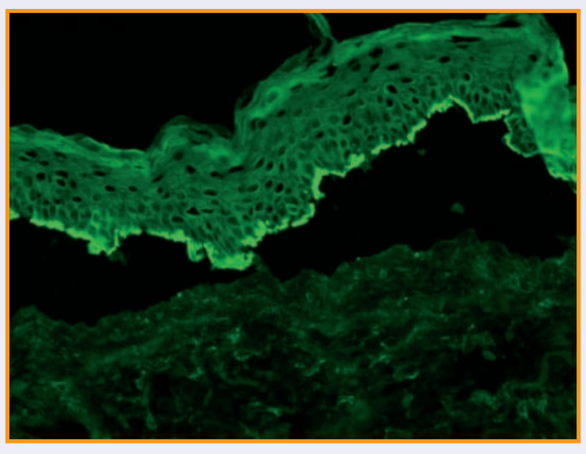

**Figure 2. Indirect immunofluorescence study showing IgG binding to the epidermal side of salt-split skin.**

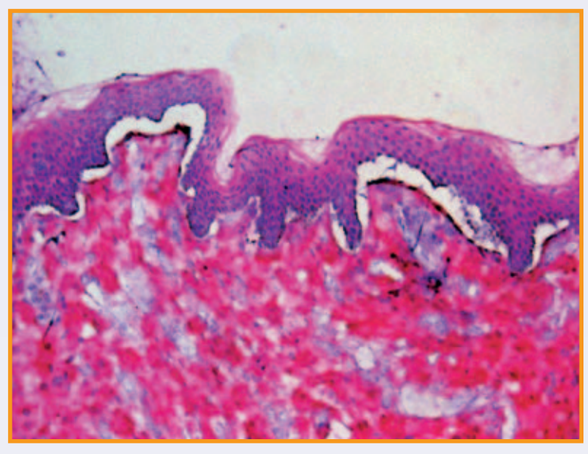

**Figure 3. Dermal-epidermal separation induced by a bullous pemphigoid patient serum co-incubated with human leukocytes form healthy donors on intact human cryopreserved skin (HE 100X).**

| Immunoblot results | Bullous pemphigoid | | | | Mucous membrane pemphigoid | | | |
|---|---|---|---|---|---|---|---|---|
| | BP230 | BP180 (180 kDa) | LAD1 (120 kDa) | LABD 97 (97 kDa) | BP230 | BP180 (180 kDa) | LAD1 (120 kDa) | LABD 97 (97 kDa) |
| Keratinocyte extracts | 47,4% | 42,1% | 21,1% | 21,1% | 15,8% | 0,0% | 26,3% | 0,0% |
| Epidermal extracts | 72,2% | 55,5% | 5,5% | 16,7% | 21,1% | 5,3% | 15,8% | 5,3% |
| Amniotic extracts | 26,3% | 36,8% | 10,5% | 0,0% | 0,0% | 0,0% | 0,0% | 0,0% |

**Table 1. Detection of circulating autoantibodies by Immunoblot on different protein extracts.**

| Fragments | Bullous pemphigoid | | Mucous membrane pemphigoid | |
|---|---|---|---|---|
| | Immunoblot | ELISA | Immunoblot | ELISA |
| **NC16A (aa 490-562)** | **73,7%** | **73,7%** | **15,0%** | **35,0%** |
| aa 915-985 | 0,0% | 10,5% | 0,0% | 0,0% |
| aa 1080-1107 | 10,5% | 5,3% | 0,0% | 5,0% |
| aa 1118-1145 | 0,0% | 10,5% | 0,0% | 5,0% |
| aa 1296-1396 | 0,0% | 0,0% | 0,0% | 30,0% |
| aa 1331-1404 | 0,0% | 15,8% | 0,0% | 5,0% |
| aa 1350-1387 | 0,0% | 0,0% | 0,0% | 0,0% |
| aa 1400-1497 | 0,0% | 10,5% | 0,0% | 0,0% |
| 4575 (aa 1365-1413) | 21,0% | 42,1% | 5,3% | 55,0% |

**Table 2. Detection of circulating autoantibodies by immunoblot and ELISA with recombinant fragments of the BP180 ectodomain.**

| Separation extent | Bullous pemphigoid | Mucous membrane pemphigoid |
|---|---|---|
| 0% | 21% | 85% |
| <25% | 32% | 15% |
| 25-50% | 21% | 0% |
| 51-75% | 16% | 0% |
| >75% | 10% | 0% |

**Table 3. Extent of dermal-epidermal separation in bullous pemphigoid and mucous membrane pemphigoid patients.**

## Discussion

The results of the indirect immunofluorescence study are comparable to previous studies. The detection of higher levels of IgA antibodies against BP180 in BP patients has been correlated with extensive mucous lesions[11]. However, none of our BP patients with IgA response presented significant mucous lesions.

The best *immunoblotting* results were obtained with epidermal and keratinocyte extracts. Amniotic extracts were by far not that sensitive. Although this tissue has some advantages as a substrate (simple protein extraction technique and high availability), in our hands, epidermal or keratinocyte extract resulted in higher detection rates.

By ELISA, circulating IgG against NC16A was detected in 74% BP versus 35% MMP individuals. On the contrary, the detection of autoantibodies against the carboxy terminal fragment (amino acids 1296-1413) was higher in MMP (65%) compared with 47% BP patients. High levels of antibodies against C-terminus portion of BP180 have been already detected in MMP patients[2-4, 12-15]. In the same way, this response to C-terminal epitopes has been related with mucosal involvement in BP, but results are still conflicting[1, 16-18].

With regard to the in vitro analysis of the blister-inducing ability, dermal-epidermal separation was much intense in BP than in MMP, likely due to the presence of higher levels of antibodies in BP sera.

## Conclusion

Most BP and MMP sera contain IgG autoantibodies against the membrane-proximal NC16A domain and, to a lesser extent, to the carboxy-terminal portion of the BP180 ectodomain. The knowledge of the specific epitopes recognized by pemphigoid autoantibodies represents the first step to further distinguish their pathogenic role separately.

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CSB Consorci Sanitari de Barcelona IMAS Institut Municipal d'Assistència Sanitària