



available at [www.sciencedirect.com](http://www.sciencedirect.com)



[www.elsevier.com/locate/yclim](http://www.elsevier.com/locate/yclim)



# Oral pemphigoid autoantibodies preferentially target BP180 ectodomain

Valentina Calabresi <sup>a</sup>, Marco Carrozzo <sup>b,\*</sup>, Emanuele Cozzani <sup>c</sup>,  
Paolo Arduino <sup>b</sup>, Giorgio Bertolusso <sup>b</sup>, Federico Tirone <sup>b</sup>, Aurora Parodi <sup>c</sup>,  
Giovanna Zambruno <sup>a</sup>, Giovanni Di Zenzo <sup>a</sup>

<sup>a</sup> Molecular and Cell Biology Laboratory, Istituto Dermatologico dell'Immacolata, IDI-IRCCS, Rome

<sup>b</sup> Department of Biomedical Sciences and Human Oncology, Oral Medicine Section, School of Medicine and Dentistry, University of Turin, Italy

<sup>c</sup> Department of Endocrinologic and Metabolic Diseases, Dermatology Section, University of Genova, Italy

Received 15 June 2006; accepted with revision 12 October 2006

Available online 1 December 2006

## KEYWORDS

Mucous membrane  
pemphigoid;  
Oral pemphigoid;  
BP180;  
Epitope profile;  
Autoantibody

**Abstract** Mucous membrane pemphigoid (MMP) comprises a heterogeneous group of autoimmune subepithelial bullous diseases very frequently having oral involvement. Very few studies have investigated the immunological status of a subset of MMP, termed oral pemphigoid (OP), presenting with exclusive oral lesions. In this study we show that 75% of 20 OP patients without scarring phenotype possessed circulating autoantibodies against the BP180 molecule, indicating a prominent role of this protein as a target antigen in OP. Of note, the frequency of reactivity against BP180 ectodomain epitopes in OP was similar to that previously reported for MMP with cicatricial phenotype, while the lack of significant recognition of BP180 intracellular domain appears to characterize OP with respect to other diseases of the pemphigoid group. Finally, the combined use of sensitive techniques allowed the detection of circulating autoantibodies in 90% of OP patients, supporting the usefulness of this approach in the diagnosis of MMP disease.

© 2006 Elsevier Inc. All rights reserved.

## Introduction

Mucous membrane pemphigoid (MMP) is a heterogeneous group of autoimmune chronic inflammatory subepithelial blistering diseases predominantly affecting mucous mem-

branes with or without skin involvement [1]. MMP is characterized by linear deposition of IgG, IgA, or C3 along the epithelial basement membrane zone (BMZ). It is recognized that scarring and loss of function are the major sequelae of the disease, except for a subset of patients with mucous membrane involvement restricted to the oral mucosa [2]. Circulating autoantibodies, detectable in a part of MMP patients, target several BMZ components including the bullous pemphigoid antigens of 180 (BP180) and 230 (BP230) kDa, the  $\alpha 6 \beta 4$  integrin, that represent three major protein constituents of the basal keratinocyte adhesion complex named hemidesmosome, and laminin 5,

\* Corresponding author. Dipartimento di Scienze Biomediche ed Oncologia Umana, Sezione di Medicina Orale, C.so Dogliotti 14, I-10126 Torino, Italy. Fax: +39 011 6633658.

E-mail address: [marco.carrozzo@unito.it](mailto:marco.carrozzo@unito.it) (M. Carrozzo).

the main laminin isoform expressed in the epithelial BMZ [2]. The most extensively characterized among MMP patients are those with cicatricial pemphigoid (CP) [3–5]. CP primarily affects mucous membranes but it may also involve the skin and is associated with scar formation. The most important target of CP autoantibodies is BP180 antigen [3–5]. This transmembrane collagenic protein that contributes to the assembly and stabilization of hemidesmosomes, consists of a globular domain located in the cytoplasm of the basal keratinocyte and a large extracellular region composed of fifteen collagen domains interrupted by sixteen noncollagenous (NC) sequences [6]. The importance of BP180 in maintenance of skin integrity is sustained by the observation that mutations in the *BP180* gene are associated with the non-Herlitz variant of junctional epidermolysis bullosa, an inherited blistering disorder characterized by skin fragility and defective dermal–epidermal adhesion [7]. The immunodominant region of the antigen, mapped in the membrane-proximal noncollagenous stretch of the ectodomain, is termed NC16A [3–5]. The frequent reactivity of CP patients autoantibodies against the C-terminal portion of BP180 has suggested that this reactivity may be responsible for the scarring phenotype [8,9]. On the other hand, a few studies have analyzed the humoral immune response in MMP exclusively involving the oral cavity, also called oral pemphigoid (OP) [10–12]. Long-term follow-up indicates that OP represents a distinct clinical subset of MMP that rarely shows scarring and is typically associated with a good prognosis [1,13]. OP autoantibodies have been reported to target the  $\alpha 6$  integrin subunit and to produce a separation of the epithelium from the basement membrane in an organ culture model [10,12]. In addition, a significant autoantibody reactivity (46%) against BP180 antigen has been detected in a group of patients with MMP predominantly affecting the oral cavity [11]. BP180 is also the major target of autoantibodies from patients affected by bullous pemphigoid (BP), the most common autoimmune subepithelial blistering disease, which predominantly affects the skin without scarring phenotype [14]. In BP, epitope profile analysis has allowed to correlate the presence of both skin and mucous membrane involvement and reactivity with epitopes in the extracellular domain (ECD) of BP180 [14,15]. These findings suggest that different BP180 epitope profiles might be associated with skin versus/or mucosal disease and also with the presence of scarring or non-scarring phenotype. In particular, delineation of correlations between the scarring sequelae and specific antigenic targets could be useful to orientate early treatment of MMP patients with exclusive oral involvement at disease onset.

In this study in order to investigate whether OP patients represent a distinct subset of MMP with respect to BP180 epitope profile, we have extensively characterized the anti-BP180 humoral immune response of 20 OP patient sera without scarring phenotype using different techniques. Considering the low titer of OP circulating autoantibodies, we have used to this purpose a panel of sensitive ELISA assays that could become a useful support for diagnosis of MMP disease in which the linear deposition of immunoglobulin and complement along the BMZ is not always easily detectable [2].

## Materials and methods

### Patient sera

A serological study of 20 random untreated patients diagnosed as having exclusive oral non-scarring pemphigoid between 1989 and 1998 has been made retrospectively. All the patients presented erosive and/or bullous oral lesions. The diagnosis was confirmed in all cases by histopathological examination which revealed subepithelial blistering, and direct immunofluorescence analysis which showed linear deposits of IgG, IgA and/or C3 at the BMZ (Table 1). All patients had a complete ophthalmological and dermatological examination. A flexible nasopharyngoscope was used to visualize the nasal mucosa, epiglottis, pharynx, larynx and proximal esophagus. All patients were followed up for almost 3 years and included in this study as OP if they had had exclusively oral lesions without scarring during the entire follow-up period. Sera from three additional OP, one CP patient with oral, esophageal and skin involvement, and one BP as control were used to affinity select a BP180 random epitope library. Sera from 55 normal healthy individuals (NHI) used as controls and

**Table 1** Oral pemphigoid patient sera reactivity in immunofluorescence and immunoblot analyses

| Patient | Age (y) | Sex | DIF          | IIF <sup>A</sup> |     | IB <sup>B</sup> |          |
|---------|---------|-----|--------------|------------------|-----|-----------------|----------|
|         |         |     |              | IgG              | IgA | IgG             | IgA      |
| OP1     | 54      | F   | IgA, IgG, C3 | +                | –   | 230             | –        |
| OP2     | 29      | F   | IgG, C3      | +                | –   | –               | –        |
| OP3     | 41      | F   | IgG, C3      | +                | +   | –               | –        |
| OP4     | 80      | M   | IgG, C3      | –                | –   | 180             | –        |
| OP5     | 71      | F   | IgG, IgA, C3 | –                | –   | 180             | –        |
| OP6     | 65      | F   | IgG, C3      | +                | –   | –               | –        |
| OP7     | 59      | F   | C3           | –                | –   | 200             | –        |
| OP8     | 90      | F   | IgG, IgA, C3 | –                | +   | 200             | –        |
| OP9     | 69      | F   | IgG, IgA, C3 | –                | –   | –               | –        |
| OP10    | 69      | F   | IgG, IgA     | –                | –   | –               | 230      |
| OP11    | 73      | F   | IgG, IgA, C3 | –                | –   | 180             | –        |
| OP12    | 80      | M   | IgG, IgA     | +                | –   | 180, 200, 230   | 180, 230 |
| OP13    | 67      | F   | IgG, C3      | –                | –   | 180             | –        |
| OP14    | 67      | F   | IgG          | +                | –   | 180, 230        | –        |
| OP15    | 81      | F   | IgG, C3      | –                | –   | –               | –        |
| OP16    | 61      | F   | IgG          | –                | –   | –               | –        |
| OP17    | 67      | F   | IgG, C3      | –                | –   | –               | –        |
| OP18    | 75      | F   | IgG, C3      | –                | –   | –               | –        |
| OP19    | 21      | F   | IgG, C3      | –                | –   | 230             | –        |
| OP20    | 61      | F   | IgG, C3      | +                | –   | 230             | –        |

<sup>A</sup>IgG and IgA titers  $\geq 1:20$  are considered positive (+) by indirect immunofluorescence labeling (IIF) on salt-split skin. <sup>B</sup>Reactivity with BP180 and BP230 using cultured keratinocyte lysates. IB: immunoblotting; DIF: direct immunofluorescence; F: female; M: male; y: year.

20 BP without mucous membrane involvement with diagnosis confirmed by direct and indirect immunofluorescence assay. The study was approved by the Ethic Committee and all patients gave informed consent.

### Indirect immunofluorescence

All OP sera and controls were diluted 1:20 and indirect immunofluorescence on human salt-split skin was performed as described [16].

### Immunoblotting

Immunoblotting (IB) was carried out using keratinocyte extracts, as described [17].

### Library selection

A BP180 random epitope library was selected using four MMP patient sera and one BP serum, as described [14].

### ELISAs based on phages displaying BP180 epitopes

Immunomicrotiter plates (Nunc, Roskilde, Denmark) were coated with rabbit anti- $\lambda$  polyclonal antibodies, in 100  $\mu$ l of 50 mM bicarbonate buffer, pH 9.6, overnight at 4°C. Wells were blocked with 200  $\mu$ l of blocking solution (5% dry no-fat milk, 0.05% Tween-20 in 1 $\times$  PBS) for 1 h at 37°C.  $5 \times 10^9$  recombinant phages displaying BP180 epitopes in 100  $\mu$ l of blocking solution were added to each well and incubated for 1 h at 37°C with shaking. Serum samples were diluted 1:100 in blocking solution and incubated, with 1  $\mu$ l bacterial extracts, 1  $\mu$ l rabbit serum, 1  $\mu$ l fetal bovine serum and wt  $\lambda$  phage ( $10^9$  pfu) for 30 min at room temperature. The mixture was then added to each well and the plates were incubated for 1 h at 37°C. After washing (0.05% Tween-20 in 1 $\times$  PBS), horseradish peroxidase-conjugated rabbit anti-human IgG (Southern Biotechnology Associates Inc, Birmingham, AL,

USA), diluted 1:20,000 in 100  $\mu$ l of blocking solution with rabbit serum (1:40) was added for 1 h at 37°C. Wells were washed and color development was achieved by using 100  $\mu$ l per well of 3,3',5,5'-tetramethylbenzidine (Sigma, St. Louis, MO, USA). After 20 min the reaction was stopped by adding 25  $\mu$ l per well of 2 M H<sub>2</sub>SO<sub>4</sub>, and OD at 450 nm, with the correlation wavelength set at 620 nm, was measured using a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Eleven epitopes displayed on phage which had been previously selected from a random epitope BP180  $\lambda$  library were employed (Fig. 1). Eight of them (AA 120–160, AA 151–170, AA 176–203, AA 282–341, AA 353–399, AA 567–622; AA 773–798, AA 915–985) had previously been assayed with BP sera [14], whereas 3 (AA 381–449, AA 1118–1145, AA 1187–1223) had never been tested before. Each serum (OP and NHI) was assayed in duplicate for the reactivity with each recombinant phage and the mean OD reading obtained with not recombinant  $\lambda$  phage was subtracted from the mean reading.

### Fusion protein production

A cDNA fragment encoding the amino acids 1400–1497 of human BP180 was amplified by PCR using the BP180 cDNA as template. The fragment was amplified with the sense 5'-ATGGATCCCACCAGGCCAGCCTG-3' and antisense primers 5'-CGGAATTCACGGCTTGACAGCAATAC-3'. The underlined sequences represent the BamHI and EcoRI restriction sites. After BamHI and EcoRI digestion, the fragment was cloned into pGEX-3X vector (Amersham Biosciences) in order to obtain GST-1400 fusion protein (Fig. 1). This recombinant protein (GST-1400) and four previously constructed GST fusion proteins, AA 316–367 [18], AA 1080–1107 [14], AA 490–562 (corresponding to the NC16A domain) [4] and AA 1331–1404 [15], were expressed in *E. coli* DH5alpha (Invitrogen, Carlsbad, CA, USA) and purified by affinity chromatography using glutathione-sepharose 4B (Amersham Biosciences) (Fig. 1).

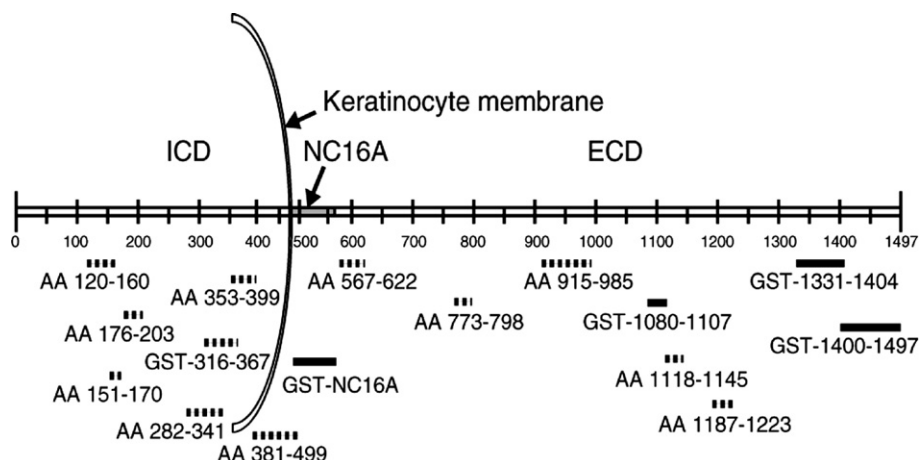


**Figure 1** Schematic diagram representing 16 BP180 epitopes used to assess reactivity of OP sera. OP-specific epitopes are shown as black lines, while epitopes recognized with comparable frequency by both OP and control sera are depicted as hatched lines. For each epitope, the amino acid residues (AA) that define the antigenic sites are indicated. GST-NC16A fusion encompasses residues 490 to 562. ICD, intracellular domain; ECD, extracellular domain.

## ELISAs based on GST fusion proteins

ELISA multiwell plates (Nunc) were coated with 800 ng of GST-NC16A or molar equivalent amounts of the other recombinant proteins in 100  $\mu$ l of 50 mM bicarbonate buffer, pH 9.6, for 2 h at room temperature. The ELISAs were performed as previously described by Mariotti et al. [15].

## Statistical analyses

Discriminating ability between patients' sera and control sera of parameters (reactivity with four GST fusion proteins and 11 phage displaying epitopes) was estimated using Area Under ROC (receiver operating characteristic) Curve (AUC). Basing on the maximization of the Youden index ( $J = \text{sensitivity} + \text{specificity} - 1$ ) the ROC curves were used to determine a cut-off value for GST fusion proteins and phage displaying epitopes ELISAs. The Fisher's Exact Probability Test was used to compare the frequencies of MMP and NHI sera reactivity to BP180 epitopes displayed on phage or produced as GST fusion protein. A  $P$  value  $\leq 0.05$  was considered significant. All statistical analyses were performed using the SPSS statistical package (SPSS, Chicago, IL, USA).

## Results

### BP180 is an important target of IgG autoantibodies in OP patients

To characterize the molecular targets of OP circulating autoantibodies we have analyzed 20 patient serum samples by indirect immunofluorescence (IIF) on salt-split skin and IB with human keratinocyte extracts (Table 1). By IIF 35% (7/20) of OP sera had IgG and 10% (2/20) IgA reacting with the epidermal side of salt-split skin. IgG and IgA never stained the dermal side of salt-split skin. By IB, six of 20 (30%) OP sera IgG reacted with BP180, five of 20 (25%) reacted with BP230 and three of 20 (15%) reacted with an antigen of 200 kDa that probably corresponds to  $\beta 4$  integrin subunit [19]. IgA reactivity to BP180 and BP230 was detected by IB in one of 20 (5%) and two of 20 (10%) OP patients, respectively (Table 1).

### MMP sera were not able to select any epitope from a BP180 random epitope $\lambda$ library

In order to identify novel epitopes recognized by MMP sera we have selected a random BP180 epitope  $\lambda$  library [14] with three OP and one CP serum samples that recognized BP180 antigen by immunoblotting or ELISA (data not shown). MMP sera were not able to select any BP180 epitope from the library, while a BP serum used as positive control selected two BP180 epitopes mapped in the NC16A domain (data not shown). This negative result could be due to the low concentration of circulating anti-BP180 antibodies in MMP sera, as compared to BP sera which had previously been shown to be able to select disease specific phages from the same random BP180 epitope  $\lambda$  library [14].

### OP patient sera react with epitopes in the BP180 ectodomain

To further characterize the reactivity of OP IgG autoantibodies against the BP180 antigen we have employed a panel of 15 antigenic sites spread over the entire BP180 sequence (Fig. 1). The reactivity of 20 OP sera and 20 BP sera without mucosal involvement against these epitopes was assessed using two types of ELISA assays, one based on BP180 epitopes produced as GST fusions and the other one based on BP180 epitopes displayed on  $\lambda$  capsid surface. Some OP patient sera reacted with BP180 intracellular domain (ICD) epitopes, but this reactivity was not significantly higher than that obtained with NHI control sera. Nine of 20 (45%) sera reacted against the BP180 ECD (Table 2). In particular, three BP180 ECD epitopes (NC16A, AA 1080–1107 and AA 1331–1404) showed a significantly higher frequency of reactivity with OP sera than with NHI control sera (Table 2). Specifically, nine of 20 (45%) sera recognized NC16A domain, three of 20 (15%) and four of 20 (20%) reacted with epitopes in the mid-portion (AA 1080–1107) and C-terminus (AA 1331–1404) of BP180, respectively (Table 2). The reactivity of BP sera against ICD and ECD epitopes was higher than that reported for OP sera (Table 2). In particular, four and three of 20 BP patient sera recognized AA 151–170 and AA 176–203 epitopes, respectively (Table 2). As to ECD epitopes 18, four, six and six of 20 BP patient sera bound NC16A, AA 915–985, AA 1080–1107 and AA 1331–1404 epitopes, respectively (Table 2).

### OP sera react with the most C-terminal portion of BP180

It has been recently reported that 15 of 40 (38%) sera of patients with CP and none of BP patient sera tested reacted with the C-terminal portion of BP180 [9] suggesting a relation between this reactivity and different clinical features of the two diseases. In order to evaluate the reactivity of our 20 non-scarring OP patient sera against the C-terminal portion of BP180 we have analyzed by IB the binding of their IgGs against the last portion (AA 1400–1497) of BP180 produced as GST fusion (GST-1400). As control, reactivity of 20 BP patient sera against this BP180 C-terminal portion was also assessed. IB was chosen because a preliminary analysis by an ELISA based on GST-1400 recombinant protein had shown an unexpected reactivity of NHI sera. Four of 20 OP (20%) reacted with the C-terminal portion of BP180 (Fig. 2), while none of 20 BP and 20 NHI sera tested reacted with GST-1400 (Table 2).

## Discussion

At least 10 different epithelial BMZ components have been reported to be recognized by MMP patients autoantibodies [2] and part of these autoantigens have been shown to be involved in MMP pathogenesis in experimental models [10,19,20]. On the other hand, there are a few immunological studies focusing on OP despite the frequent observation of this subset of MMP [21,22].

In our study combining very sensitive techniques, circulating autoantibodies against BMZ antigens could be detected in 90% of OP patients. This finding strongly suggests that the

**Table 2** Reactivity by ELISA and immunoblotting of 20 OP sera against 16 BP180 antigenic sites<sup>A</sup>

| OP SERA       | Intracellular domain |                   |                   |       |                    |       |       | Extracellular domain |       |       |                   |                   |        |        |                   |                   |
|---------------|----------------------|-------------------|-------------------|-------|--------------------|-------|-------|----------------------|-------|-------|-------------------|-------------------|--------|--------|-------------------|-------------------|
|               | E-120 <sup>B</sup>   | E-151             | E-176             | E-282 | G-316 <sup>C</sup> | E-353 | E-381 | G-NC16A              | E-567 | E-773 | E-915             | G-1080            | E-1118 | E-1187 | G-1331            | G-1400            |
| OP1           | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | +                 | –      | –      | +                 | +                 |
| OP2           | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP3           | +                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | +                 | –      | –      | –                 | –                 |
| OP4           | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP5           | +                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP6           | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | +                 | –      | –      | –                 | +                 |
| OP7           | –                    | –                 | –                 | –     | –                  | –     | +     | +                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP8           | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP9           | –                    | –                 | –                 | –     | –                  | –     | +     | –                    | –     | –     | +                 | –                 | –      | –      | –                 | –                 |
| OP10          | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP11          | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP12          | –                    | –                 | –                 | –     | +                  | +     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP13          | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP14          | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP15          | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | –                 | –      | –      | +                 | +                 |
| OP16          | –                    | –                 | +                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP17          | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | –                 | +      | –      | +                 | –                 |
| OP18          | –                    | –                 | –                 | –     | –                  | –     | –     | –                    | –     | –     | –                 | –                 | –      | –      | –                 | +                 |
| OP19          | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | –                 | –      | –      | –                 | –                 |
| OP20          | –                    | –                 | –                 | –     | –                  | –     | –     | +                    | –     | –     | –                 | –                 | –      | –      | +                 | –                 |
| Positive sera | 2/20 <sup>D</sup>    | 0/20              | 1/20              | 0/20  | 1/20               | 1/20  | 2/20  | 9/20 <sup>E</sup>    | 0/20  | 0/20  | 1/20              | 3/20 <sup>E</sup> | 1/20   | 0/20   | 4/20 <sup>E</sup> | 4/20 <sup>E</sup> |
| BP sera       | 1/20                 | 4/20 <sup>E</sup> | 3/20 <sup>E</sup> | 2/20  | 1/20               | 0/20  | 2/20  | 18/20 <sup>E</sup>   | 2/20  | 1/20  | 4/20 <sup>E</sup> | 6/20 <sup>E</sup> | 0/20   | 0/20   | 6/20 <sup>E</sup> | 0/20              |
| NHI sera      | 2/55                 | 1/55              | 1/55              | 2/55  | 3/55               | 1/55  | 2/55  | 0/55                 | 0/55  | 0/55  | 1/55              | 1/55              | 1/55   | 0/55   | 1/55              | 0/20              |

<sup>A</sup>Reactivity of OP, BP and NHI sera against BP180 epitopes has been assessed by ELISA, while reactivity against the epitope G-1400 has been tested by immunoblotting. <sup>B</sup>E corresponds to peptides displayed on the capsid surface. <sup>C</sup>G corresponds to peptides produced as GST-fusion proteins. <sup>D</sup>Number of sera reactive/total number of sera tested. <sup>E</sup>Denotes a statistically significant difference in reactivity between patients with OP or BP and NHI sera ( $p \leq 0.05$  by Fisher's). E-120: AA 120–160; E-151: AA 151–170; E-176: AA 176–203; E-282: AA 282–341; G-316: GST-316–367; E-353: AA 353–399; E-381: AA 381–449; G-NC16A: GST-490–562; E-567: AA 567–622; E-773: AA 773–798; E-915: AA 915–985; G-1080: GST-1080–1107; E-1118: AA 1118–1145; E-1187: AA 1187–1223; G-1331: GST-1331–1404; G-1400: GST 1400–1497.

reported low frequency of detection of circulating autoantibodies in the sera of OP patients is mainly due to insufficiently sensitive techniques rather than to unknown targeted autoantigens. Moreover, considering IB and ELISA

reactivity together, 75% of the OP patients had circulating autoantibodies against BP180 antigen indicating that this molecule represents a major target antigen in MMP patients with exclusive non-scarring oral lesions. A recent study on a

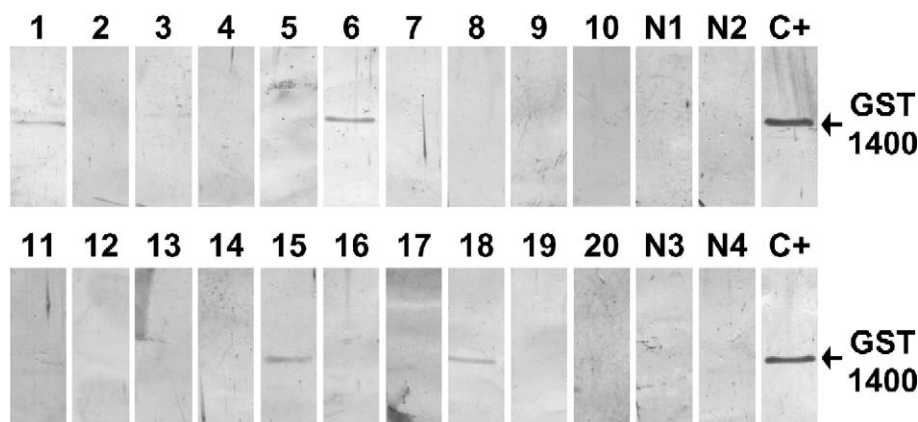


**Figure 2** Reactivity of 20 OP patient sera against the C-terminal portion of BP180 antigen by immunoblotting. OP sera were preabsorbed with GST protein and tested for reactivity against the prokaryotic recombinant fusion protein GST-1400 (GST-1400–1497). Lanes: 1–20, OP1–OP20; N1–N4, control sera from normal healthy individuals; C+, MMP patient serum that strongly reacts with GST-1400 used as positive control.

large cohort of MMP with scarring phenotype and involvement of various mucosal sites also reported that BP180 is the major autoantigen recognized by these patients [23]. Altogether these data show that BP180 reactivity is a key immunological feature shared by MMP with oral but also multiple mucosal site involvement with or without cicatricial phenotype.

On the other hand, we were not able to identify in our OP cohort the previously described reactivity against a ~120 kDa band indicating the presence of autoantibodies against the  $\alpha 6$  integrin subunit [10,12,24], whereas three patients had autoantibodies against an antigen of ~200 kDa that could correspond to  $\beta 4$  integrin [19]. This different result could be due to the different substrates (bovine gingival lysate and prostate carcinoma DU145 cells versus epidermal keratinocyte extracts) or lysate preparation conditions or detection methods employed.

Previous studies have demonstrated that IgA autoantibodies specific for BP180 ectodomain are typically detected in CP but not in BP patient sera [5,25]. Furthermore, in CP patients the presence of both circulating IgG and IgA anti-BMZ antibodies has been associated with more severe and persistent disease manifestations [26]. In the present study, although seven of 20 OP patients had IgA deposition at the BMZ, only few had circulating IgA reacting with BP180 antigen by both immunoblotting (1/20) and indirect immunofluorescence (2/20), suggesting that IgA autoantibodies may not play a major role in the pathogenesis of OP disease.

As to the epitope pattern of BP180, OP patients sera did not significantly recognize ICD of the antigen. This lack of reactivity appears to distinguish OP from both BP, that significantly recognized E-151 and E-176 in the BP180 ICD, and CP which have been previously demonstrated to significantly target BP180 ICD epitopes [5]. On the other hand, most of the OP reactivity was directed against epitopes in the BP180 ectodomain, similarly to what reported for CP [4]. Indeed, four BP180 ECD epitopes (NC16A, AA 1080–1107, AA 1331–1404 and AA 1400–1497) showed a higher reactivity with OP sera than with NHI sera. Of note, four of 20 OP patients (20%) had circulating autoantibodies against the most C-terminal portion of BP180 (AA 1400–1497) and six of 20 OP (30%) reacted at least with one of two C-terminal antigenic sites tested (AA 1331–1404 and/or AA 1400–1497), indicating that previously described reactivity of CP patients against this BP180 region [4,9] may be not specifically related with the cicatricial phenotype that is absent in our patients. On the other hand, the lack of reactivity of 20 BP sera without mucosal involvement against GST-1400 is in agreement with previously reported data and suggests a possible relation between the recognition of the C-terminal portion of BP180 and the mucous membrane involvement typically present in MMP patients. In addition, OP sera displayed a low frequency of reactivity against the NC16A domain (45%). This reactivity is similar to previously reported data on CP patients [4,5] and appears to distinguish MMP from BP which is characterized by a high frequency of NC16A recognition [15] and a different clinical presentation. Interestingly, five of six OP sera that reacted with BP180 by IB on keratinocyte extracts were negative by ELISA, suggesting the existence of additional BP180 antigenic sites that are not represented in

our panel of epitopes. These epitopes could be dependent on antigen denaturation or generated by post-translational modifications of the BP180 molecule. In conclusion, the frequency of reactivity (50%) against BP180 ECD in our non-scarring OP patient population appears similar to that previously reported for CP patients [4,5], while the lack of significant recognition of BP180 ICD appears to characterize OP with respect to both BP and CP [5,14]. Altogether these results highlight the importance of BP180 ectodomain as target of OP autoantibodies and suggest that the peculiar clinical phenotype may be related to a reactivity against specific regions of BP180.

Finally, up to 90% of OP patients were positive combining very sensitive techniques such as indirect immunofluorescence on salt-split skin, immunoblotting on keratinocyte extracts and ELISA assays for detection of anti-BP180 IgGs, suggesting a role of this approach as valuable support for diagnosis of MMP disease. Furthermore, the identification of BP180 epitopes recognized by OP patients could be relevant for the design of innovative therapies based on specific immunoadsorption strategies.

## Acknowledgments

This work was supported by the Italian Ministry of the Education, University and Research, Italian Ministry of Health, Department of Biomedical Sciences Human Oncology, University of Turin and a grant from the European Community (QLG1-CT-2001-02007).

## References

- [1] C. Scully, M. Carrozzo, S. Gandolfo, P. Puiatti, R. Monteil, Update on mucous membrane pemphigoid: a heterogeneous immune-mediated subepithelial blistering entity, *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endo.* 88 (1999) 56–68.
- [2] L.S. Chan, A.R. Ahmed, G.J. Anhalt, W. Bernauer, K.D. Cooper, M.J. Elder, J.D. Fine, C.S. Foster, R. Ghohestani, T. Hashimoto, T. Hoang-Xuan, G. Kirtschig, N.J. Korman, S. Lightman, F. Lozada-Nur, M.P. Marinkovich, B.J. Mondino, C. Prost-Squarcioni, R.S. Rogers III, J.F. Setterfield, D.P. West, F. Wojnarowska, D.T. Woodley, K.B. Yancey, D. Zillikens, J.J. Zone, The first international consensus on mucous membrane pemphigoid: definition, diagnostic criteria, pathogenic factors, medical treatment, and prognostic indicators, *Arch. Dermatol.* 138 (2002) 370–379.
- [3] S.D. Balding, C. Prost, L.A. Diaz, P. Bernard, C. Bedane, D. Aberdam, G.J. Giudice, Cicatricial pemphigoid autoantibodies react with multiple sites on the BP180 extracellular domain, *J. Invest. Dermatol.* 106 (1996) 141–146.
- [4] H. Murakami, S. Nishioka, J. Setterfield, B.S. Bhogal, M.M. Black, D. Zillikens, K.B. Yancey, S.D. Balding, G.J. Giudice, L.A. Diaz, T. Nishikawa, C. Kiyokawa, T. Hashimoto, Analysis of antigens targeted by circulating IgG and IgA autoantibodies in 50 patients with cicatricial pemphigoid, *J. Dermatol. Sci.* 17 (1998) 39–44.
- [5] E. Schmidt, C. Skrobek, A. Kromminga, T. Hashimoto, G. Messer, E.B. Bocker, K.B. Yancey, D. Zillikens, Cicatricial pemphigoid: IgA and IgG autoantibodies target epitopes on both intra- and extracellular domains of bullous pemphigoid antigen 180, *Br. J. Dermatol.* 145 (2001) 778–783.
- [6] G.J. Giudice, D.J. Emery, L.A. Diaz, Cloning and primary structural analysis of the bullous pemphigoid autoantigen BP180, *J. Invest. Dermatol.* 99 (1992) 243–250.

- [7] J.A. McGrath, B. Gatalica, A.M. Christiano, K. Li, K. Owaribe, J. R. McMillan, R.A. Eady, J. Uitto, Mutations in the 180-kD bullous pemphigoid antigen (BPAG2), a hemidesmosomal transmembrane collagen (COL17A1), in generalized atrophic benign epidermolysis bullosa, *Nat. Genet.* 11 (1995) 83–86.
- [8] C. Bedane, J.R. McMillan, S.D. Balding, P. Bernard, C. Prost, J.M. Bonnetblanc, L.A. Diaz, R.A. Eady, G.J. Giudice, Bullous pemphigoid and cicatricial pemphigoid autoantibodies react with ultrastructurally separable epitopes on the BP180 ectodomain: evidence that BP180 spans the lamina lucida, *J. Invest. Dermatol.* 108 (1997) 901–907.
- [9] J.B. Lee, Y. Liu, T. Hashimoto, Cicatricial pemphigoid sera specifically react with the most C-terminal portion of BP180, *J. Dermatol. Sci.* 32 (2003) 59–64.
- [10] K.C. Bhol, L. Goss, S. Kumari, J.E. Colon, A.R. Ahmed, Autoantibodies to human alpha6 integrin in patients with oral pemphigoid, *J. Dent. Res.* 80 (2001) 1711–1715.
- [11] M. Carrozzo, E. Cozzani, R. Brocchetto, M. Carbone, M. Pentenero, P. Arduino, A. Parodi, S. Gandolfo, Analysis of antigens targeted by circulating IgG and IgA antibodies in patients with mucous membrane pemphigoid predominantly affecting the oral cavity, *J. Periodontol.* 75 (2004) 1302–1308.
- [12] K.A. Rashid, J.N. Stern, A.R. Ahmed, Identification of an epitope within human integrin [alpha]6 subunit for the binding of autoantibody and its role in basement membrane separation in oral pemphigoid, *J. Immunol.* 176 (2006) 1968–1977.
- [13] N. Mobini, N. Nagarwalla, A.R. Ahmed, Oral pemphigoid. Subset of cicatricial pemphigoid? *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endo.* 85 (1998) 37–43.
- [14] G. Di Zenzo, F. Grosso, M. Terracina, F. Mariotti, O. De Pita, K. Owaribe, A. Mastrogriaco, F. Sera, L. Borradori, G. Zambruno, Characterization of the anti-BP180 autoantibody reactivity profile and epitope mapping in bullous pemphigoid patients, *J. Invest. Dermatol.* 122 (2004) 103–110.
- [15] F. Mariotti, F. Grosso, M. Terracina, M. Ruffelli, P. Cordiali-Fei, F. Sera, G. Zambruno, A. Mastrogriaco, G. Di Zenzo, Development of a novel ELISA system for detection of anti-BP180 IgG and characterization of autoantibody profile in bullous pemphigoid patients, *Br. J. Dermatol.* 151 (2004) 1004–1010.
- [16] W.R. Gammon, R.A. Briggaman, A.O. Inman III, L.L. Queen, C.E. Wheeler, Differentiating anti-lamina lucida and anti-sublamina densa anti-BMZ antibodies by indirect immunofluorescence on 1.0 M sodium chloride-separated skin, *J. Invest. Dermatol.* 82 (1984) 139–144.
- [17] E. Cozzani, J. Kanitakis, J.F. Nicolas, D. Schmitt, J. Thivolet, Comparative study of indirect immunofluorescence and immunoblotting for the diagnosis of autoimmune pemphigus, *Arch. Dermatol. Res.* 286 (1994) 295–299.
- [18] G. Di Zenzo, V. Calabresi, F. Grosso, M. Caproni, M. Ruffelli, G. Zambruno, The intracellular and extracellular domains of BP180 antigen comprise novel epitopes targeted by pemphigoid gestationis autoantibodies, *J. Invest. Dermatol.* (2006).
- [19] K.C. Bhol, J.E. Colon, A.R. Ahmed, Autoantibody in mucous membrane pemphigoid binds to an intracellular epitope on human beta4 integrin and causes basement membrane zone separation in oral mucosa in an organ culture model, *J. Invest. Dermatol.* 120 (2003) 701–702.
- [20] Z. Lazarova, C. Yee, T. Darling, R.A. Briggaman, K.B. Yancey, Passive transfer of anti-laminin 5 antibodies induces subepidermal blisters in neonatal mice, *J. Clin. Invest.* 98 (1996) 1509–1518.
- [21] G. Shklar, P.L. McCarthy, Oral lesions of mucous membrane pemphigoid. A study of 85 cases, *Arch. Otolaryngol.* 93 (1971) 354–364.
- [22] G. Laskaris, A. Sklavounou, J. Stratigos, Bullous pemphigoid, cicatricial pemphigoid, and pemphigus vulgaris. A comparative clinical survey of 278 cases, *Oral Surg. Oral Med. Oral Pathol.* 54 (1982) 656–662.
- [23] N. Oyama, J.F. Setterfield, A.M. Powell, Y. Sakuma-Oyama, S. Albert, B.S. Bhogal, R.W. Vaughan, F. Kaneko, S.J. Challacombe, M.M. Black, Bullous pemphigoid antigen II (BP180) and its soluble extracellular domains are major autoantigens in mucous membrane pemphigoid: the pathogenic relevance to HLA class II alleles and disease severity, *Br. J. Dermatol.* 154 (2006) 90–98.
- [24] M. Mignogna, A. Lanza, L. Rossiello, V. Ruocco, A.R. Ahmed, Comparison of reactivity and epitope recognition between sera from American and Italian patients with oral pemphigoid, *Clin. Exp. Immunol.* 145 (2006) 28–35.
- [25] Z. Nie, T. Hashimoto, IgA antibodies of cicatricial pemphigoid sera specifically react with C-terminus of BP180, *J. Invest. Dermatol.* 112 (1999) 254–255.
- [26] J. Setterfield, P.J. Shirlaw, B.S. Bhogal, K. Tilling, S.J. Challacombe, M.M. Black, Cicatricial pemphigoid: serial titres of circulating IgG and IgA antibodies correlate with disease activity, *Br. J. Dermatol.* 140 (1999) 645–650.