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Autoreactive T and B Cells from Bullous Pemphigoid (BP) Patients Recognize Epitopes Clustered in Distinct Regions of BP180 and BP230

Sybille Thoma-Uszynski, Wolfgang Uter, Susanne Schwietzke, Gerold Schuler, Luca Borradori, and Michael Hertl

Bullous pemphigoid (BP) is the most frequent autoimmune subepidermal blistering disease of the skin. Clinical features of BP include generalized, tense blisters on normal and erythematous skin and, in some cases, blisters and erosions of the mucous membranes (1, 2). A hallmark of BP is the presence of circulating autoantibodies against BP180 and BP230, two components of junctional adhesion complexes called hemidesmosomes, critically involved in the maintenance of dermoepidermal adhesion (1, 3–10).

In BP, immunologic injury mediated by autoantibody and/or autoreactive Th cells results in an impairment of the dermoepidermal junction integrity with subepidermal blister formation. Specifically, both in vitro and in vivo studies showed that the autantibody-mediated tissue damage of BP largely depends on complement activation and subsequent chemotactic recruitment of inflammatory cells and secretion of proteases (11–13). Autoantibody pathogenicity may also be due to direct inhibition of the targeted adhesion molecules upon binding to sequential and/or conformational epitopes.

BP180 is a transmembrane protein with a large collagenous extracellular domain (ECD) serving as cell adhesion molecule, whereas BP230 is a cytoplasmic protein critically involved in intermediate filament anchorage to the cell membrane (8). The noncollagenous NC16A region adjacent to the transmembrane part on the BP180 ectodomain represents an immunodominant region harboring major autoantibody epitopes recognized not only in BP, but also in related bullous autoimmune diseases such as pemphigoid gestationis (Pg) and mucous membrane pemphigoid (MMP) (14–17). This is in line with the recent ex vivo isolation of BP180-NC16A-reactive B cells from the peripheral blood of BP patients (18). However, autoantibodies also target epitopes located outside the NC16A domain in the COOH terminus and the central portion of the BP180 ECD (19–22).

Th cells are presumably involved in early disease development and perpetuation of acquired autoantibody-mediated autoimmune bullous diseases, although the exact molecular mechanisms of specific T cell activation in BP remain elusive. Upon proper costimulation, Th cells become activated and secrete distinct cytokines, which stimulate B cells and thus foster plasma cell development and autoantibody production. Autoreactive Th cells that recognize regions of the BP180 ECD, including the immunodominant NC16A region, have been described in selected patients with BP and Pg (23–27). Clinically, BP has been associated with several MHC class II alleles, HLA-DQB1*0301, -*0305, -*0602, and -*0603 (28). HLA-DQB1*0301, in particular, has been linked to
IgG autoantibody production in patients with MMP (29). In addition, a previous study from our laboratory demonstrated that the activation of autoreactive BP180-reactive Th cells from a cohort of HLA-DQβ1*0301−BP patients was indeed restricted by this BP-associated HLA class II allele (23).

Detailed knowledge of autoreactive T cell and B cell responses in BP may facilitate the development of specific immunomodulatory therapeutic strategies. The purpose of this study was to investigate the BP180- and BP230-specific epitope recognition of autoreactive Th and B cells in patients, and to compare epitope recognition of the cellular and humoral autoimmune response in patients with active BP. Our findings demonstrate that both BP180 and BP230 contain distinct regions that are recognized to a similar extent by autoreactive Th and B cells. Noteworthy, T and B cell reactivity against BP180, but not against BP230, was detected in the majority of BP patients, suggesting that BP180 is the primary autoantigen of BP.

Materials and Methods

Patients and controls

This analysis includes 35 patients with BP and 32 age- and sex-matched controls with unrelated skin conditions such as herpes zoster, erysipelas, atopic dermatitis, or eczema. Blood samples were obtained from patients with clinically active BP (n = 35), i.e., patients with newly diagnosed BP or who had not yet received immunosuppressive treatment. Patients and volunteers gave written consent to participate in this study, which was adherent to the Declaration of Helsinki Guidelines and has been approved by the local institutional review board.

Diagnosis of BP was based on the following criteria: 1) typical clinical presentation with tense cutaneous blisters; 2) histopathological evidence of subepidermal blister formation; 3) linear IgG and/or C3 deposits at the dermoeipidermal 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 skin or IgG reactivity with BP180 or BP230). Patients and volunteers gave written consent to participate in this study, which was adherent to the Declaration of Helsinki Guidelines and has been approved by the local institutional review board.

Expression and purification of BP180 and BP230 recombinant proteins

The recombinant BP proteins were produced in a baculovirus expression system as GST and 6His-tagged fusion proteins as described below (Fig. 1); cDNA expression constructs for human BP180 and BP230 were generated as reported previously (30, 31). In brief, cDNAs for BP180 or BP230 were used as a template to amplify by PFU DNA polymerase with forward and reverse primers containing either an EcoRI or a NotI site the desired target sequences of BP180 and BP230 (Stratagene). The amplified cDNA fragments were subsequently subcloned into the pAcGHLT-A baculovirus transfer vector (BD Pharmingen) by ligation of double-digested PCR fragments into the corresponding cut vectors. Correctness of both constructs was verified by sequencing. pAcGHLT-A vectors were cotransfected with Baculo-DNA (SixPack; BD Clontech) into Sf21 insect cells, and recombinant baculoviruses were amplified using a previously described protocol (32). The recombinant proteins and the GST/6His control protein (generated by cotransfection of an empty pAcGHLT-A vector) were expressed in Sf21 cells as described previously (33). BP180 and BP230 recombinant proteins were purified as follows: three days after infection, 10 x 10^6 baculovirus-infected insect cells were suspended in 1 ml of 6 M guanidine-hydrochloride, incubated for 24 h at −20 °C, and then centrifuged (2000 x g, 15 min). Solubilized proteins were purified by affinity chromatography over Nickel-NTA agarose (Qiagen) using the batch method according to the manufacturer’s protocol. The BP180 and BP230 recombinant proteins and the GST/6His control protein were purified under denaturing conditions by adding 6 M guanidine-hydrochloride to the lysis buffer and 8 M urea to washing and elution buffers. Finally, purified proteins were gradually dialyzed against PBS supplemented with 8 M to 3 M urea at 4 °C for 48 h. Affinity-purified proteins were stored in aliquots (at −80 °C). Protein concentrations were determined by a commercial kit (DC Protein Assay; Bio-Rad) according to a modified protocol by Lowry. BP180ex (baculoprotein containing the entire ECD of BP180; aa residues 485–1497), BP180-N1 (baculoprotein of BP180; aa residues 490–812), BP230-N2 (baculoprotein containing the NH2-terminus of BP230; aa residues 1–1307), BP180-M (baculoprotein of BP180; aa residues 809–1106), BP180-C1 (baculoprotein of BP180; aa residues 1048–1465), BP180-C2 (baculoprotein of BP180; aa residues 1352–1465), BP230-C1 (baculoprotein containing the COOH-terminus (aa residues 1881–2649) of BP230), BP230-C2 (baculoprotein containing the COOH-terminus (aa residues 2077–2649) of BP230), and BP230-N were fractionated by 10% SDS-PAGE and visualized by Coomassie staining and Western blot with a monoclonal mouse anti-GST Ab (1:2000, clone GST3-4C; Zymed) to verify m.w. and immunoreactivity (Figs. 1 and 2).

ELISA analysis using BP230 and BP180 recombinant proteins

ELISA procedures were performed as described recently (10, 34). Optimal conditions for the ELISA were established by various checkerboard titrations. The affinity-purified BP230 and BP180 proteins were immobilized on 96-well polystyrene plates (Maxisorb Immunoplate; Nunc) by coating each well with 0.5 μg of BP230-N, BP230-C1, BP230-C2, and BP230ex, BP180-N1, BP180-N2, BP180-C1 (baculoprotein of BP180; aa residues 467–567), BP180-C2 (baculoprotein of BP180; aa residues 1352–1465), BP180-M, BP180-C1, and BP180-C2, respectively, or a molar equivalent amount of the control protein GST/6His in 100 μl of 0.1 M bicarbonate buffer (pH 9.8), at 4 °C for 5 h. ELISA plates were then washed five times with TTBS (PBS (pH 7.5) with 0.05% Tween 20; Merck) and blocked for 2 h with 100 μl of 5% skimmed milk powder in TTBS at room temperature. BP and control sera were diluted 50-fold in blocking buffer and added to the wells in duplicate. After an incubation period of 12 h at 4 °C and consecutive washes, each well was reacted with alkaline phosphatase-labeled goat anti-human IgG (γ)(1/6000; Kirkegaard & Perry Laboratories). After another series of washes, 100 μl of p-nitrophenylphosphate (Kirkegaard & Perry Laboratories) were added to the wells, and OD was measured at 405 nm when a defined patient serum (positive reference control)
One NH2-terminal construct (BP230-N, containing residues 2077–2649) and one NH2-terminal construct (BP230-C1, containing residues 1881–2649 and BP230-C2, containing residues 2077–2649) and one NH2-terminal construct (BP230-N, residues 1–1307) were used. The purified BP230 proteins were transferred onto nitrocellulose membrane and reacted with an anti-GST Ab.

reached an OD of 1.0 as determined by a Wallac 1420 microplate reader (Wallac).

All extinction data were standardized based on the positive and negative internal standards, which were set to 1 and 0 OD units, respectively. Background reactivity of IgG to the control protein GST/6XHis was subtracted from the mean OD values obtained with BP180-N and BP180-C proteins. To evaluate plate-to-plate variability, each plate included identical internal standards, which were set to 1 and 0 OD units, respectively. Background reactivity (Table I).

PBMC from BP patients were isolated by Ficoll centrifugation. A total of 10^5 cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 2 mM penicillin, 50 µg/ml streptomycin (Invitrogen Life Technologies), and 10% human serum (PAA Laboratories) were plated in triplicate in round-bottom microtiter plates. BP180 and BP230 recombinant proteins were added at a final concentration of 10 µg/ml. PHA (1%) was used as a positive control, and GST at 10 µg/ml was used as mock control. After 6 days, each well was pulsed with 0.5 µCi [3H]thymidine for 12 h, and incorporation was measured with a scintillation counter.

BP180- or BP230-specific T cell proliferation was expressed as a stimulation index (SI), which is the ratio of [3H]thymidine uptake in cultures with Ag and cultures without Ag. SI values >2.5 were considered positive.

Statistical analysis

The focus of this study is a comparison between results obtained with 1) two different assays yielding quantitative data and 2) different epitopes. ELISA results (OD values) had a skewed distribution, which was scarcely amenable to log transformation, and were thus tested with nonparametric statistical methods, e.g., Wilcoxon-Mann-Whitney or Kruskal-Wallis test for overall equality of mean scores. SI values followed a skewed distribution, even after log transformation, and were treated in an identical fashion (the log transformation was used to rescale SI values for better graphical representation; this transformation has no influence on the rank statistics used).

The distribution of ELISA data is shown as “box plot”: the box shows first and third quartile, the median (as line) and the arithmetic mean (as dot), the whiskers represent values below the first quartile and above the third quartile within the 1.5-fold interquartile range, respectively, and outliers beyond the whiskers are shown as squares. The correlation between ELISA and SI results obtained with the same epitope, and between related epitopes (BP180-C2 and BP180-C1 and BP180-N1 and BP180-N2, respectively) obtained with the same assay was quantified with the Spearman rank correlation coefficient.

For data analysis, the statistical software package SAS, version 8.2 (SAS Institute), was used. Receiver operating characteristic curves were compiled by additionally using a freeware SAS macro package (35) to evaluate the diagnostic properties of different IgG Abs to BP180 Ags with respect to correctly classifying cases of BP. Because there are no “natural” cutoffs for these Ab levels, and because there are no previous studies that had provided evidence concerning an appropriate diagnostic threshold (except in case of BP 230 and BP180ex 10), an “optimum” cutoff was determined data-adaptively by choosing the OD values with the largest Youden Index (YI = sensitivity + specificity – 1).

For those analyses requiring dichotomization of SI results as positive and negative, values >2.5 were considered positive. For dichotomization of ELISA results as positive and negative, cutoffs were defined based on the YIs obtained with the different BP180 and BP230 recombinants (see Table II).

Results

Clinical and immunoserological phenotype of the studied BP patients

The present analysis involved 35 patients with acute BP. None of the patients received topical or systemic immunosuppressive treatment at the time of blood sampling. Among the 35 BP patients, 12 had limited and 23 extensive involvement. Mucosal involvement was observed in five BP patients, two of whom had limited disease. All patients’ sera were reactive with human saline split skin (97% reactive with the epidermal site of the blister) by indirect immunofluorescence (IIF) microscopy (Table I). Overall, 94.2 and 80% of the studied BP patients had anti-BP180 and anti-BP230 IgG autoantibody, respectively. Thus, a relatively homogeneous patient cohort was available for detailed analyses regarding BP180- and BP230-specific T cell proliferative responses and IgG reactivity (Table I).

IgG autoreactivity with BP180 and BP230

Several recombinant forms of BP180 and BP230 known to harbor B cell epitopes were successfully produced in a baculovirus expression system and subsequently affinity purified. The recombinant proteins, the apparent m.w. of which corresponded to that predicted on the basis of their cDNA, were immunoreactive with appropriate mAbs directed against the GST-tag (Figs. 1 and 2) and were expressed in an identical fashion in the expression system and subsequently affinity purified. The recombinant proteins, the apparent m.w. of which corresponded to that predicted on the basis of their cDNA, were immunoreactive with appropriate mAbs directed against the GST-tag (Figs. 1 and 2) and were used for ELISA analysis.

Using the BP180 and BP230 recombinants for ELISA analysis, the diagnostic performance was best regarding IgG reactivity against the entire BP180 ECD (BP180ex; >90%) followed by IgG reactivity against NH2-, COOH-terminal, and midportion of the

Table I. Clinical and immunological characteristics of BP patients and healthy control subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>21 of 35</td>
<td>22 of 32</td>
</tr>
<tr>
<td>Age (min-median-max)</td>
<td>46–76–94</td>
<td>13–72–89</td>
</tr>
<tr>
<td>Involvement: limited</td>
<td>12 of 35</td>
<td>None</td>
</tr>
<tr>
<td>extensive</td>
<td>23 of 35</td>
<td>None</td>
</tr>
<tr>
<td>IIF (SSS)</td>
<td>35</td>
<td>NT</td>
</tr>
<tr>
<td>Epidermal</td>
<td>31</td>
<td>NT</td>
</tr>
<tr>
<td>Dermal + epidermal</td>
<td>3</td>
<td>NT</td>
</tr>
<tr>
<td>Dermal</td>
<td>1</td>
<td>NT</td>
</tr>
<tr>
<td>Anti-BP180 IgG only</td>
<td>7 of 35</td>
<td>None</td>
</tr>
<tr>
<td>Anti-BP230 IgG only</td>
<td>2 of 35</td>
<td>None</td>
</tr>
<tr>
<td>Anti-BP180 and anti-BP230 IgG</td>
<td>26 of 35</td>
<td>None</td>
</tr>
</tbody>
</table>

*a Min, Minimum; Max, maximum; SSS, saline split skin; NT, not tested.*
BP180 ECD (Fig. 3 and Table II). The majority, e.g., 29 of 35 (82.8%), of the BP patients’ sera preferentially recognized the NH2-terminal portion (BP180-N2), whereas only about one-third of the BP patients had detectable IgG against the midportion of the BP180 ECD (BP180-M) (Fig. 3 and Table III). The COOH-terminal part of the BP180 ECD covered by the recombinant proteins BP180-C1 and BP180-C2 was a target for IgG autoantibodies of almost 75% of the studied BP patients (Fig. 3 and Table III). IgG reactivity against BP230 was seen in >70% of the studied BP patients, with the NH2 terminus being recognized by 72.4% of patients’ sera and the COOH terminus in >65.4% of the patients (Fig. 3 and Table IV). Due to the smaller number of BP230-reactive patients, their clinical phenotype could not be related to BP230 epitope recognition.

IgG reactivity against the recombinant BP180 proteins was analyzed regarding clinical severity. Almost all patients with limited and extensive disease had detectable IgG against BP180, particularly against the recombinant proteins encompassing the NH2-terminal portion of the BP180 ECD (Table III). The NH2 terminus including the immunodominant NC16A domain (BP180-N2) was recognized by 86.9% of the BP patients with extensive and by 75% of the patients with limited disease (p = 0.39, Fisher’s exact test, 2-sided). In addition, most patients had detectable IgG directed against the COOH-terminal recombinant proteins of BP180 ranging from 73.9% to 83.3% without significant differences between the clinical categories. The midportion of the BP180 ECD (BP180-M) was more frequently recognized by specific IgG in limited BP (50%) than in extensive BP (30.4%) (Table III). There was, however, no direct relationship between a distinct autoantibody profile and the involvement of mucous membranes in the studied BP patients. Noteworthy, there is no sequential homology between the NH2 terminus, midportion, and COOH terminus of the BP180 ectodomain (6).

**T cell recognition of BP180 of the BP patients**

This study was restricted to patients with acute onset BP before treatment with systemic glucocorticoids and adjuvants because of in vitro detection of proliferative T cell responses to BP180 was strongly compromised by immunosuppressive treatment (data not shown). The majority (28 of 35; 80%) of the studied BP patients developed a significant proliferative T cell response to the ECD of BP180 (Table III). The NH2-terminal portion of the BP180 ECD (BP180-N1/N2) including the NC16A region was most frequently (71.4–77.1%) recognized, followed by the COOH-terminal BP180-C1/C2 (62.8–65.7%) and the midportions of the ECD of BP180 (BP180-M; 57.1%) (Table III).

With regard to the clinical phenotype, almost 74% of the BP patients with extensive disease showed T cell responses against the NH2-terminal regions of the ECD of BP180, 56.5% to the COOH-terminal regions, and 47.8% BP patients had also autoaggressive T cells specific for the midportion of BP180 (Table III). Noteworthy, all of the five BP patients with an exclusive T cellular response against the NC16A domain showed an extensive phenotype with numerous blisters/erosions on the trunk. In contrast, BP patients with limited disease showed less frequent T cell response against the NH2-terminal region of the ECD (66.7%), but an increased T cell response against midportion (75%) and COOH terminus (83.3%) of BP180. However, all of these differences were nonsignificant (p > 0.15, Fisher’s exact test).

**Comparison of BP180-specific T cell and B cell reactivity**

Based on the hypothesis that T cell and IgG reactivities are directly linked, we analyzed the correlation of T cell and B cell responses against distinct regions of BP180 and BP230 (Tables III and IV). The majority of the BP patients showed autoreactive T cell and IgG reactivity against the ECD of BP180 (BP180ex; 71.4%). This association was strongest when reactivity with the NC16A region (BP180-N2; 71.4%) was assessed, compared with that with the COOH terminus (BP180-C1/C2; 54.3–57.1%) and midportion of BP180 (BP180-M; 51.4%) (Table III) (Fig. 4).

IgG reactivity against the recombinant proteins BP180-N1 (aa 490–812) and BP180-N2 (aa 467–567) was strongly correlated (0.98; p < 0.0001; BP180-N1 vs BP180-N2; Fig. 5A), an observation suggesting that the major epitope(s) was located within the NC16A region (aa 489–566), which represents exactly the overlapping region between BP180-N1 and BP180-N2. In addition, T cell reactivity against these overlapping BP180 recombinants encompassing the NH2-terminal region of the BP180 ECD revealed a high degree of correlation, providing support to the idea of the

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**Table II. Clinical severity of BP and T cell and IgG reactivity against distinct regions of BP180**

<table>
<thead>
<tr>
<th>Extensive Disease</th>
<th>Limited Disease</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (%) T cell/IgG reactivity</td>
<td>Positive (%) T cell/IgG reactivity</td>
<td>Positive (%) T cell/IgG reactivity</td>
</tr>
<tr>
<td>BP180ex</td>
<td>19 of 23 (82.6)/18 of 23 (78.3)</td>
<td>9 of 12 (75.0)/11 of 12 (91.7)</td>
</tr>
<tr>
<td>BP180-N1</td>
<td>17 of 23 (73.4)/17 of 23 (73.9)</td>
<td>8 of 12 (66.7)/6 of 12 (50.0)</td>
</tr>
<tr>
<td>BP180-N2</td>
<td>17 of 23 (73.4)/20 of 23 (86.9)</td>
<td>8 of 12 (66.7)/9 of 12 (75.0)</td>
</tr>
<tr>
<td>BP180-M</td>
<td>11 of 23 (47.8)/7 of 23 (30.4)</td>
<td>9 of 12 (75.0)/6 of 12 (50.0)</td>
</tr>
<tr>
<td>BP180-C2</td>
<td>13 of 23 (56.5)/17 of 23 (73.9)</td>
<td>10 of 12 (83.3)/9 of 12 (75.0)</td>
</tr>
<tr>
<td>BP180-C1</td>
<td>13 of 23 (56.5)/17 of 23 (73.9)</td>
<td>9 of 12 (75.0)/10 of 12 (83.3)</td>
</tr>
</tbody>
</table>
existence of T cell epitopes within the NC16A region of BP180 (Fig. 5C). On the same line, IgG and T cell reactivity against the two overlapping COOH-terminal BP180 recombinants, BP180-C2 and BP180-C1, was strongly correlated (0.71; p < 0.0001), suggesting that T and B cell epitopes were located in the same regions (Fig. 5B). Thus, the major relevant T cell and B cell epitopes of BP180 are presumably harbored within residues aa 467–567 (BP180-N2) and residues aa 1352–1465 (BP180-C2), respectively.

Recognition of BP230 by autoreactive T and B cells

Sixteen BP patients were further analyzed for BP230-specific autoreactive T cell responses. Seven of 16 (43.8%) and 6 of 16 (37.5%) patients had IgG autoantibody reactive with the COOH terminus and the NH₂ terminus of BP230, respectively, with 8 of 16 (50%) serum samples showing anti-BP230 IgG reactivity (Table IV). Overall, 9 of 16 (56.3%) BP230-IgG-reactive patients showed a T cellular response to COOH terminus and/or NH₂ terminus of BP230. Correlation of BP230-specific T cell proliferation and IgG reactivity was weak to negligible for any of the investigated BP230 recombinant proteins (Fig. 6).

When the concordance of BP180 and BP230-specific IgG reactivity was analyzed, 43.8% of the BP patients (7 of 16) had both BP180- and BP230-specific IgG, whereas 43.8% of the sera were exclusively anti-BP180 IgG reactive (Table V). Seven of the 16 (43.8%) BP patients showed both T and IgG reactivity against the NH₂ and/or COOH terminus of BP230 (Table IV). Noteworthy, the majority (14 of 16) of the studied patients showed proliferative T cell responses to BP180 including the aforementioned BP230-reactive patients (Table IV).

Discussion

In this study, we present a comprehensive analysis of T and B cell recognition of BP180 and BP230, the autoantigens of the potentially devastating autoimmune bullous disorder, BP. Our results show that the vast majority of the 35 studied BP patients analyzed had both autoaggressive T cells and IgG autoantibody reactive with defined extracellular regions of BP180 and, to a lesser extent, with distinct NH₂- and COOH-terminal domains of BP230. This finding is of particular interest in light of the current discussion as to whether the transmembranous adhesion molecule, BP180, or the intracellular hemidesmosomal component, BP230, is the major autoantigen of BP.

In extension to previous investigations, this study provides evidence that autoaggressive T cells recognize three major regions of the ECD of BP180, i.e., NH₂ terminus, midportion, and COOH terminus. Previous reports only identified serum autoantibody (10, 21, 33, 34) and autoaggressive T cells (23) recognizing epitopes within the NH₂-terminal region of the BP180 ECD in the majority
of BP patients. Specifically, these studies focused on T cell recognition of the NC16A subdomain, which resides within the NH2-terminal region of BP180 (24, 25, 27, 36). In fact, a considerable number of BP and Pg patients studied displayed NC16A-specific peripheral T cell responses, which were mainly of the Th2 type or of a mixed Th1/Th2 type (24, 25, 27, 36). Specifically, Lin et al. (24) identified autoreactive T cells directed against the NC16A region of the BP180 ECD in 12 patients with active BP and two patients with Pg. Because the NC16A region also represents the major binding site for autoantibodies in BP, we concluded that this region contained immunodominant epitopes for both autoaggressive T and B cells in BP. This contention is supported by the observation that IgG autoantibodies against the NC16A are pathogenic in a mouse model of BP (11, 12). Leyendeckers et al. (18) recently demonstrated that BP180-NC16A-reactive B cells are present in the peripheral blood of the majority of BP patients. T cell recognition of BP180-NC16A-reactive B cells are present in the peripheral blood of the majority of BP patients. T cell recognition of BP180-NC16A seems to be rather heterogeneous because BP180-NC16A-specific T cell clones from BP patients preferentially expressed TCRBV13, whereas T cell clones derived from a Pg patient expressed TCRBV3 (26). Recently, IFN-γ secretion by NC16A-specific autoreactive T cells was detected in 2 of 10 patients with MMP and in 2 of 17 patients with BP, but not in healthy controls (27). These results are in apparent contrast to a previous study from our group showing that BP180-reactive T cells from BP patients preferentially secreted Th2-like cytokines, whereas BP180-responsive T cell clones from healthy individuals secreted Th1-like cytokines, such as IFN-γ (23).

In contrast to the aforementioned studies, the present study demonstrates that both autoreactive Th cells and IgG autoantibodies not only recognize epitopes located within the NC16A region, but also within the COOH-terminal and central portion of the ECD of BP180. Although the NC16A region turned out to be a major T cell target in our analyses, the COOH terminus of BP180 was recognized both by T cells and autoantibodies in up to 65 and 75% of the studied BP patients, respectively. Pathogenetically, this BP180 region is of particular interest, because IgG reactivity with the COOH terminus has been associated with a distinct clinical phenotype of BP presenting with a preferential mucosal involvement (21, 34, 37). T cells responsive to the COOH terminus had not yet been identified. Unexpectedly, the midportion of the ECD of BP180 was recognized by T cells in 57.1% of the BP patients, whereas autoantibody reactivity against this region was only observed in

![FIGURE 4. T and B cell reactivity against distinct regions of the BP180 ectodomain. T cells and sera derived from 35 patients with acute onset BP were cocultured with the BP180 recombinants or reacted by ELISA, respectively. BP180-specific IgG reactivity (OD values at 405 nm) and T cell proliferation (BP180-specific SI) are displayed as dot plots. SI was defined as the ratio of [3H]thymidine uptake in T cell cultures with/without Ag and was considered to be significant at a value ≥2.5.](http://www.jimmunol.org/)
37% of the patients. This antigenic region of the BP180 ECD had been identified as the exclusive target of a subgroup of patients with BP (20). This observation implies that, in BP, relevant B cell epitopes are present in three distinct regions, i.e., NH₂ terminus, COOH terminus, and the central portion of the ECD of BP180.

When BP180-specific T cell responses and IgG reactivity in individual BP patients were compared, there was a correlation of at least 50% for the various BP180 recombinant proteins. The NH₂-terminal portion of the BP180 ECD was targeted by T and B cells to the greatest extent, whereas an overlapping T and B cell response to midportion and COOH terminus of BP180 was only

![FIGURE 5](image)

Correlation of IgG against overlapping proteins of the NH₂ terminus (A) and COOH terminus (B) was relatively high (BP180-N1 vs BP180-N2, p < 0.0001; and BP180-C1 vs BP180-C2, p < 0.0001). Similarly, T cell reactivity against overlapping NH₂- (C) and COOH-terminal (D) BP180 proteins was relatively high (BP180-N1 vs BP180-N2, p < 0.0001; and BP180-C1 vs BP180-C2, p < 0.0001).

![FIGURE 6](image)

BP230-specific IgG reactivity (OD values at 405 nm) and proliferative T cell responses (as determined as a SI) were investigated in 16 patients with acute onset BP. SI was defined as the ratio of [³H]thymidine uptake in T cell cultures with/without Ag and was considered to be significant at a value ≥2.5. Data are displayed as dot plots for the different BP230 recombinant proteins. Correlation of BP230-specific IgG and T cell proliferation was moderate.
seen in >50% of the patients (Table III). This finding is in line with a previous study by Lin et al. (25) who detected both autoantibody and T cell reactivity directed against the NC16A-domain of BP180 in the majority of the patients investigated.

A similar degree of overlapping T and B cell recognition was also seen in most of the BP patients for the COOH-terminal region of BP180. The midportion of the BP180 ECD was more frequently recognized by autoreactive T cells than by IgG autoantibodies. Moreover, T and B cell recognition of the midportion of BP180 was seen more frequently in patients with limited than in patients with extensive disease (41.7 vs 13%) (Table III). Thus, differential BP180 epitope recognition may be associated with limited vs extensive BP.

As shown in Fig. 7, there is a body of evidence demonstrating that both T and B cell epitopes are located in defined and restricted regions of the ectodomain of BP180. Specifically, a recent study by di Zenzo et al. (21) has shown by phage display library screening that IgG autoantibodies recognized epitopes clustered in the three aforementioned regions of the BP180 ectodomain. Ongoing studies will have to define whether T and B cell epitopes of BP180 are indeed identical, as partly shown for the NC16A domain, or just clustered in distinct regions.

A major finding of the present study was the identification of autoaggressive T cells that targeted distinct regions of BP230, the second major autoantigen of BP. The current understanding of the pathogenesis of BP supports the concept that IgG against BP230 are not the primary pathogenic autoantibody but may enhance the local inflammatory response that precedes the autoantibody-induced loss of dermoeidermal adhesion (2, 7). Autoreactive T cells and/or IgG autoantibodies against the NH2- and COOH-terminal regions of BP230 were detectable in ~40% of the tested BP patients. In ~50% of the studied patients, both autoaggressive T and B cells recognized identical regions, i.e., the NH2- and COOH-terminal rod domains of BP230. The majority of the patients with BP230-specific T cellular responses had also BP230-reactive IgG in their sera. Noteworthy, >90% of the BP patients, including those with BP230-reactive T cells, had also T cells specific for BP180. A single BP patient was neither responsive to BP180 on the T cellular level nor showed a proliferative T cell response to BP230. Despite extensive clinical data, no relationship could be established between T and B cell recognition of BP230 and a distinct clinical phenotype. Recent studies from our group provided statistical evidence that the titers of BP230-specific IgG were related to a more limited phenotype of BP, whereas the extent of IgG reactivity against the NH2 terminus of BP180 was associated with extensive BP (10, 34).

Noteworthy, recent studies in pemphigus vulgaris, an unrelated autoimmune bullous skin disorder, showed that autoaggressive T cells specific for desmoglein 3 preferentially targeted regions located in the NH2-terminal domains EC1 and EC2 (38) that were also preferentially recognized by IgG autoantibodies (39). In a HLA class II-transgenic mouse model of myasthenia gravis, autoreactive T cells responded to three cytoplasmic peptide sequences of the human acetylcholine receptor, which were also targeted by the pathogenic autoantibodies (40). Together, these findings strongly suggest that both Th and B cells recognize similar or identical regions of the autoantigens of several human autoimmune disorders.

In summary, the present findings demonstrate that BP180 and, to a lesser extent, BP230 are major targets for both autoreactive T cells and IgG autoantibodies in active BP. With regard to BP180 recognition, autoreactive T cells and IgG autoantibodies target similar or identical antigenic regions within the NH2 and COOH termini and the midportion of the ECD of BP180, with the NC16A domain.

### Table V. Concordance between T cella and IgGd reactivity against BP180 and BP230

<table>
<thead>
<tr>
<th>BP230a,b</th>
<th>pos.</th>
<th>neg.</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP180a,b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pos.</td>
<td>9/7</td>
<td>6/7</td>
<td>15/14</td>
</tr>
<tr>
<td>neg.</td>
<td>0/1</td>
<td>1/1</td>
<td>1/2</td>
</tr>
</tbody>
</table>

a [3H]Thymidine uptake (3-HT) was calculated as a SI (3-HT in cultures with/without Ag). A SI > 2.5 was considered to be significant, pos: positive, neg: negative.

domain being the immunodominant region. Noteworthy, preferen-
tial T and B cell recognition of the NH2-terminal and midportion of BP180 seemed to be associated with extensive and limited BP, respectively. In addition, T and B cell recognition of BP230 was focused on the NH2- and COOH-terminal rod domains. Finally, our observations support the concept that BP180 is the primary autoantigen of BP.

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Disclosures
The authors have no financial conflict of interest.

References


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