Human Monoclonal Autoantibodies Specific for the Bullous Pemphigoid Antigen 1 (BPAg 1)

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Bullous pemphigoid (BP) is an acquired blistering skin disease associated with the production of IgG autoantibodies to the 230-kDa BP Ag (BPAg1). To better characterize the epitopes of BPAg1, we generated immortalized B cell lines secreting human mAbs (HumAbs) to BPAg1 from two BP patients whose sera reacted with native BPAg1 but not with a recombinant BP55 carboxyl-terminal peptide. Ab-producing B cell lines were established by EBV infection of CD40-activated PBMCs. Three independent clonal lines were obtained that secreted IgG HumAbs, including one IgG1κ (BP3) and two distinct IgG4κ (BPI and BP2). These three HumAbs immunoprecipitated BPAg1. Blocking immunofluorescence experiments and phylogenetic studies showed that these Abs recognize different epitopes of BPAg1. This analysis with HumAbs further extends the serologic demonstration of the wide variety of epitopes recognized by BP mAbs which contrasts with the limited number of epitopes recognized by thyroid peroxidase monoclonal autoantibodies.

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Bullous pemphigoid (BP) is an autoimmune bullous skin disorder associated with production of autoantibodies to components of hemidesmosomes (HDs), namely the BP Ags of molecular mass 230 kDa and 180 kDa (1–3). Immunochemical experiments have shown that Abs to the 230-kDa BP Ag (BPAg1) were found in a majority of BP sera and BPAg1 was thus referred to as the major BP Ag (4). Recently, cDNA was isolated that codes for a protein of 2606 amino acids with 10 putative sites for N-glycosylation (3–5). Further molecular studies have documented a monoallelic gene localized on chromosome 6 at locus p11-p12 (6). At the present time, relatively little is known about the antigenic peptides recognized by BP autoantibodies. Most studies aimed at their determination were focused on the reactivity of BP patients’ sera or of rabbit immune sera with fusion proteins or with synthetic peptides localized at the carboxy-terminal end of BPAg1 (7, 8). These studies showed that BP patients’ sera contain autoantibodies reactive with the C-terminal portion of BPAg1. Interestingly, a human mAb (HumAb) (MoAb-SE-HY-4B) recognizes the carboxyl-terminal portion of the BPAg1 (9, 10). In this context, we have recently demonstrated that 98 of 120 of BP sera bind to a 55-kDa recombinant Escherichia coli-derived polypeptide (rBP55) encoded by the carboxyl-terminal portion of BPAg1 cDNA (Gaucherand, M., and J. F. Nicolas, submitted) suggesting that anti-BPAg1 Abs from the other 22 patients are directed either against the amino-terminal part of the native BPAg1 or to a conformational carbohydrate epitope of the protein. To characterize these Abs, we generated cell lines producing monoclonal autoantibodies from two of these patients.

Although the generation of immortalized human B cell lines producing HumAbs has proved to be difficult, recent progress in culturing human B lymphocytes has now facilitated this. In particular, we have shown that human lymphocytes can undergo long term proliferate when cultured in the presence of CDw32 (FcγRI)–transfected fibroblastic L cells with anti-CD40 and IL-4 (11, 12). The
HumAbs specific for BPAgl. Thus we generated three cloned B cell lines producing three IgG isolated HumAbs from BP patients with unconventional autoantibodies to BPAgl. The present report describes the combination of the CD40 system (composed of the CDw32-L cells and anti-CD40) and EBV was recently shown to allow the generation of immortalized B cell lines and clones producing IgG and IgA HumAbs with a high frequency (F. Rousset, personal communication). Thus we isolated HumAbs from BP patients with unconventional autoantibodies to BPAg1. The present report describes the generation of three cloned B cell lines producing three IgG HumAbs specific for BPAg1.

Materials and Methods

Patients

PBMCs were obtained from two patients with the typical clinical, histologic, and immunopathologic criteria of BP (Table I). Both patients presented an active but mild BP disease, with the presence of erythematous plaques and few bullae on the arms, the legs, and the trunk. They were untreated at the time of blood sampling. Both patients presented clinical improvement of their dermatosis following topical corticosteroid therapy. The two patients’ sera were negative by immunoblotting on a BP recombinant protein of 55 kDa molecular mass (rBP55), which was obtained from a cDNA sequence encoding the carboxyl-terminal region of the 230-kDa BP Ag.

Lymphocyte preparation

Heparinized peripheral blood (50 ml) was obtained by venipuncture. Mononuclear cells were isolated on Ficoll density gradient and washed three times with PBS before use.

Establishment of B cell lines

For each patient, B cell lines were derived from PBMCs by EBV infection alone (13) or by a combination of EBV infection and CD40 activation (11). For EBV infection of B cells PBMCs (10^7/ml) were incubated for 2 h at 37°C with regular gentle shaking with 10-fold concentrated EBV-containing supernatant from the B95-8 cell line (14,15). PBMCs were then washed in Iscove’s modified medium (16) supplemented with 10% FCS, t-glutamine, and gentamycin, and cultured at a density of 10^5 cells/well with 200 µg/ml of cyclosporin A in 48-well plates (Costar, Cambridge, MA). EBV-transfected B cell lines obtained from the two patients were maintained in culture for 3 wk and supernatants were then tested for anti-basement membrane one (BMZ) reactivity by IF on skin cryostat sections.

Generation of B cell lines using CD40-mediated activation of B cells has been previously described (11). After EBV infection, PBMCs (10^5 cells/well) were cocultured for 2 wk in 48-well plates (Costar) with 2.5 × 10^6 irradiated mouse fibroblastic Ltk^- cells transfected with the human CD32 molecule and 0.5 µg/ml of the anti-CD40 mAb Mab89 (17). The B cell line supernatants were then tested for anti-BMZ activity in an indirect IF assay.

Production of B cell clones

B cell lines that demonstrated anti-BMZ activity in their supernatants were subsequently cloned by limiting dilution in round-bottom microtiter wells. Cells were plated at 1, 3, and 9 cells/well. Irradiated allogeneic PBMCs (2 500 rad) were added at day 0 (10^5 cells/well) and at day 15 (2.5 × 10^5 cells/well). The clones were grown for 3 to 4 wk. The culture supernatants were then tested for their IgG anti-BMZ activity. The anti-BMZ-producing clones were expanded and analyzed by FACS for light chain expression and by ELISA for Ig isotype production and subclasses.

Purification and fluoresceinination of the Abs

IgG fractions were isolated from the supernatants of the B cell lines by 30% saturated ammonium sulfate precipitation, followed by protein G4b fast-flow (P-3296, Sigma Chemical Co., Saint Quentin Fallavier, France) affinity chromatography. The chelate was dialyzed against PBS and concentrated to 1 mg/ml. HumAbs were disulfide-oxidized overnight at 4°C against a solution of carbonan/dibasic buffer (PH 9.3) and then incubated for 30 mins at 37°C with an FITC solution (F7250, Sigma Chemical Co., F7250) diluted to 3 mg/ml in DMSO. FITC-conjugated Ab was separated from free FITC on a 2 ml G25 column (Columns RD-10 Sephadex G25 M, Pharmacia, Uppsala, Sweden) equilibrated with PBS containing 0.5 M NaCl and 0.5% NaN3.

Immunohistochemistry

Indirect immunohistochemical analyses were performed according to previously described methods (18). Briefly, supernatants from the cell cultures and purified Abs were tested on 4 µm cryostat tissue sections of rabbit lip, normal human skin and salt-split human skin (1.0 M NaCl). The specific binding was revealed using FITC-conjugated goat anti-human IgG (Zymed, San Francisco, CA) or peroxidase-conjugated goat anti-human IgG (Dako LSAB, Denmark). For the cloning step, only B cell lines with a BMZ staining were kept for further analysis. In some IF competition experiments on skin sections, FITC-conjugated mouse anti-human IgG1 and IgG4 (Immunootech S.A., Lumilly, France) were used.

For immunoperoxidase electron microscopy (IEM), cryostat sections (10 µm) of normal human skin plunge-frozen in liquid propane were labeled, before embedding in Epon, with the HumAb-containing supernatant using indirect immunoperoxidase staining with goat anti-human IgG (F(ab')2)-peroxidase conjugate (Tago, CA) (19, 20). A normal human serum and a BP serum recognizing the 230-kDa BP Ag on immunoblot were used as negative and positive controls, respectively. Ultrathin sections were observed without counterstaining.

Immunochemical analysis

Immunoprecipitation of the 230-kDa BP Ag was done from [35S]amino acid-labeled keratinocytes as described earlier (2) using protein A-Sepharose (CL-4B Pharmacia, France). The patients’ sera and purified HumAbs (1 mg/ml) were used undiluted. Immunoprecipitated material was subjected to 5% to 10% gradient SDS-PAGE, and the reaction was developed on an X-OMAT Kodak film exposed for 4 wk. The immunoblot assay was performed from epidermal and dermal extracts as previously described (21).

Results

Establishment of lymphoblastoid cell lines and clones producing anti-BMZ Abs

B cell lines were derived from PBMCs of two BP patients, either with classical EBV infection or with EBV infection associated with CD40-mediated B cell activation. These B cell lines were maintained in culture 3 and 2 wk, respectively, before indirect IF assay of the culture supernatants

| Table 1. Characteristics of the two BP patients’ sera |
|-------------------------------|----------------|
|                               | Patient 1 | Patient 2 |
| Direct IF*                    | IgG       | IgG       |
| Indirect IF                   | 1,280     | 2,560     |
| Highest dilution             | + roof    | + roof    |
| Immunoblot                   | +         | +         |
| rBP55 Immunoblot             | –         | –         |
| Immunoprecipitation           | +         | +         |

* Deposit of IgG at the dermal-epidermal junction on inflammatory peribulgeous skin.

** Immunoblot reactivity on epidermal (cultured normal human keratinocytes) protein extract.

† Immunoblot reactivity on the rBP55 protein.

‡ Immunoprecipitation reactivity on [35S]amino acid-labeled keratinocytes.
for anti-BMZ IgG activity using normal human skin sections. This difference regarding the duration of the culture is explained by the more rapid growth of cells in the CD40 system, which is caused by the higher EBV infection rate of anti-CD40-activated B cells (F. Rousset, unpublished results). As shown in Table II, anti-BMZ IgG Ab-producing B cell lines were obtained only when cells were cultured in the CD40 system (14 anti-BMZ producing B cell lines from 96, vs none out of 96). Consistent with the skin reactivity of the sera from these two patients, no B cell lines producing HumAbs binding to other skin components were identified. The single positive B cell line obtained from patient 1 was subsequently cloned by limiting dilution, resulting in 73 of 768 wells producing anti-BMZ IgG Abs. Only one clone producing HumAb anti-BPAgl (BP1) was kept for further analysis (BP1). From patient 2, 13 B cell lines were positive for anti-BMZ IgG Abs at the initial screening. Eight of these positive B cell lines were cloned and four yielded B cell clones producing anti-BMZ HumAbs (BP2, BP3, BP4, BP5); two of these HumAbs were demonstrated to be specific (see below) for BPAgl (BP2, BP3).

**Characterization of HumAbs to BMZ Ags**

As shown in Table III and consistent with the initial screening based on the use of an FITC-labeled specific anti-IgG, the three anti-BPAgl HumAbs were IgG. One was IgG1 (BP3) and two were IgG4 (BP1 and BP2). The light chain was κ in all three cases. The reactivity of the three anti-BPAgl HumAbs to components of the BMZ was identical to that of natural BP autoantibodies. Immunohistologic analysis shows that the three HumAbs labeled the dermal-epidermal junction in a variety of species including humans, rabbits (Fig. 1a), and mice. These HumAbs bind specifically to components of the BMZ of ectodermic-derived epithelia as found in the skin, esophagus, tongue, and lip. They all stained an Ag found at the epidermal side of salt-split human skin (Fig. 1b). Finally, the three HumAbs gave a polarized staining of the basal pole of cultured keratinocytes.

**Table II. B cell lines and B cell clones producing anti-BMZ and anti-BPAgl Abs**

<table>
<thead>
<tr>
<th>Patient 1</th>
<th>Patient 2</th>
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<tbody>
<tr>
<td><strong>EBV</strong></td>
<td><strong>EBV</strong></td>
</tr>
<tr>
<td>+ CD40</td>
<td>+ CD40</td>
</tr>
<tr>
<td>Anti-BMZ lines</td>
<td>0</td>
</tr>
<tr>
<td>Anti-BMZ clones</td>
<td>0</td>
</tr>
<tr>
<td>Anti-BMZ clones</td>
<td>0</td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>HumAb</strong></th>
<th><strong>Patient 1</strong></th>
<th><strong>Patient 2</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>BP1/P1</td>
<td>IgG4</td>
<td>κ</td>
</tr>
<tr>
<td>BP2/P2</td>
<td>IgG4</td>
<td>κ</td>
</tr>
<tr>
<td>BP3/P3</td>
<td>IgG1</td>
<td>κ</td>
</tr>
</tbody>
</table>

| **Characteristics of the anti-BPAgl HumAbs**

The cutaneous BMZ is composed of multiple components (for a review, see Ref. 22). To further delineate the target Ags of these HumAbs, indirect immunoelectron microscopy was performed on normal human skin and showed that the three HumAbs bound to HDs (Fig. 2). The staining was restricted to the intracellular portion of the structure and no reactivity was found in the lamina lucida or in any other portion of the BMZ. No labeling was observed at the keratinocyte plasma membrane outside the hemidesmosomal plaque.

**Immunochromal characterization of the Ag recognized within the HDs by the HumAbs**

The three HumAbs are specific for native BPAgl.

**Table III. Characteristics of the anti-BPAgl HumAbs**

<table>
<thead>
<tr>
<th>HumAb/Patient</th>
<th>Patient 1</th>
<th>Patient 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isotype</strong></td>
<td><strong>Light chain</strong></td>
<td><strong>Immunoblot</strong></td>
</tr>
<tr>
<td>BPAgl</td>
<td>IgG4</td>
<td>κ</td>
</tr>
<tr>
<td>BPAgl</td>
<td>IgG4</td>
<td>κ</td>
</tr>
<tr>
<td>BPAgl</td>
<td>IgG1</td>
<td>κ</td>
</tr>
</tbody>
</table>

* *a* 4.8 x 10⁶ PBMCs were EBV infected. PBMCs were seeded at 1 x 10⁶ cells/well in 48-well Costar plates (see Materials and Methods).
* *b* 12.0 x 10⁶ PBMCs were EBV infected, and then seeded as in footnote a.

* *a* Indirect immunofluorescence.
* *b* Immunoelectron microscopy.

**Competition IF tests**

The epitope specificity of the three anti-BPAgl HumAbs was analyzed by the ability of each of the three unconjugated HumAbs to inhibit binding of homologous or heterologous FITC-conjugated HumAbs on normal human skin cryostat sections. BMZ staining of all FITC-HumAbs was blocked by pretreatment of the homologous unlabeled HumAb. The three HumAbs did not compete for binding of each other, indicating that they recognize distinct epitopes. In addition, preincubation of skin sections with
FIGURE 1. Indirect IF reactivity of one anti-BPAG1 HumAb (BP3) on rabbit lip (a) and on salt-split human skin (b). All HumAbs reacted in the same way and labeled the epidermal side of salt-split skin. The faint dot pattern staining of the dermis is nonspecific and observed also with the anti-human IgG conjugate alone. Bar = 10 μm.

Patient's serum blocked the binding of HumAbs established from the corresponding patient, suggesting that the relevant B cell clone was present in vivo and did not arise as a result of in vitro selection. As expected, preincubation of skin sections with the unconjugated IgG1 or IgG4 HumAbs did not inhibit binding of IgG4 or IgG1 Abs, respectively, present in BP sera.

Antibodies produced by injection of rabbits with rBP55 did not block the staining of any of the HumAbs, thus confirming that they are specific for distinct epitopes.
**FIGURE 2.** Ultrastructural localization of the Ag recognized by the BP3 anti-BPAG1 HumAb in normal human skin. Indirect immunoperoxidase pre-embedding staining revealed a specific reactivity of the HumAb in the basal pole of basal layer keratinocytes (a) with a dotted pattern of staining that represents HDs. The same pattern was observed with a BP serum positive for the 230-kDa BP Ag (b). Note the absence of staining in a basal melanocyte (open arrow). No counterstaining. n = nucleus. Bar = 500 nm.

**Differential reactivity of the three anti-BPAG1 HumAbs to BMZ components in phylogenetic studies**

Phylogenetic studies were conducted to more precisely characterize the BPAG1 epitopes recognized by the three HumAbs. The phylogenetic groups tested here included mammals (human, monkey, mice, rats, and ferret), birds, reptile, batrachians, and fishes. The sera from these two BP patients bound the skin BMZ of the species tested from each group. BP3 was specific for an epitope common to the BP Ags found in mammals, birds, and reptiles. The reactivity of BP1 and BP2 was restricted to mammals and both recognized human, monkey, mouse and rat skin. Thus, as shown in Figure 4, two distinct patterns of reactivity were found indicating the existence of at least two B cell epitopes on amino-terminal part of BPAG1. Indeed, the lack of cross-competition between BP1 and BP3 indicates the presence of a third epitope. Furthermore, considering the reactivity of the human serum with batrachians and fishes and the lack of reactivity of the HumAbs on tissues from these two groups, a fourth epitope is suggested.

**FIGURE 3.** Immunochemical analysis of the reactivity of the five anti-BMZ HumAbs using immunoprecipitation (a) and immunoblot (b). BP1, BP2, and BP3 immunoprecipitated the BP Ag 1, but only BP3 immunoblotted the 230-kDa BP Ag. Lanes 1–5: HumAbs BP1 to BP5. Lane 6 and lane 7: Sera from the two BP patients (P1 and P2). Lane 8: Serum from a healthy age-matched person.

**Discussion**

The aim of this study was to generate a set of human monoclonal IgG autoantibodies reacting with the cutaneous BMZ of patients suffering from the skin-blistering disease bullous pemphigoid. As a HumAb reacting with a recombinant form of the carboxyl-terminal portion of the molecule had already been described (10), we focused our attention on Abs directed against BPAG1 but that would not react with such a polypeptide. This is particularly relevant as we had found patients whose serum recognized natural BPAG1 but did not react by immunoblotting with rBP55 protein, a recombinant protein obtained from the cDNA that encoded the carboxyl-terminal portion of BPAG1.

Preliminary attempts, using only EBV transformation of blood cells of patients suffering from bullous pemphigoid did not yield B cell lines producing Abs reacting with the BMZ (23), and accordingly, in the present study, EBV transformation alone did not yield any line producing BMZ-specific Abs. In sharp contrast, the combination of EBV transformation and CD40, earlier shown to result in strong and long lasting B cell proliferation (F. Rouset, unpublished results), yielded one cell line from the first patient and 13 cell lines from the second patient. Five of the 14 lines established from the two patients yielded
The BP3 HumAb exhibited a unique reactivity. The two BP antigens, BP1 and BP2, gave the same pattern of staining and were specific for BPAgl because they immunoprecipitated it. Interestingly, the phylogenetic study shows that we can distinguish two groups of HumAbs: two HumAbs (BP1 and BP2) staining an epitope present on mammalian tissues only and one HumAb (BP3) recognizing an epitope found on mammalian as well as nonmammalian skin (birds and reptiles). BP sera have previously been found to immunostain skin from animals such as fishes, amphibians, reptiles, birds, and mammals. In summary, the three human monoclonal autoantibodies generated in this present study confirm the heterogeneity of human autoantibody binding sites on BPAgl. This heterogeneity contrasts with the restricted specificity of autoantibodies to thyroid peroxidase found in patients with autoimmune thyroid disease (26).

**References**


**FIGURE 4.** Phylogenetic studies. Indirect IF reactivity of the anti-BPAgl HumAbs on skin sections from various animal groups. Five groups were tested, namely mammals, birds, reptiles, batrachians, and fishes. In the figure, each line represents a group and each open circle represents a positive staining of the BMZ in the given groups. Two HumAbs, BP1 and BP2, gave the same pattern of reactivity (R2), whereas the BP3 HumAb exhibited a unique reactivity. The two BP patient sera stain all tissue tested (R1).

clones that produced HumAbs reacting particularly strongly with the BMZ and binding to HDs. These clones stably produced HumAbs over a period of at least 6 mo. The other B cell lines lost their reactivity during the cloning phase. Three HumAbs (BP1, BP2, and BP3) proved to be specific for BPAgl because they immunoprecipitated it. The three B cell lines (two of which were derived from different patients) exhibited distinct immunochemo-phylogenetic characteristics and most likely correspond to three independent B cell clones. Thus, one IgG1 and two IgG4 Abs were found, in accordance with the IgG1 and IgG4 subclass distribution of anti-BMZ autoantibodies detected in BP patients' sera (24, 25). Cross-competition IF experiments for binding to the BMZ indicated that BP1 and BP2 bound to two epitopes of BPAgl, which are detected in man and ferret but not in cat or nonmammals. BP3 bound to a quite distinct epitope because there was no cross-competition between it (BP3) and BP1 or BP2 for binding to the basal membrane and because it was able to bind to the BMZ of mammals, birds and reptiles.

The presence of multiple B cell epitopes on BPAgl was previously suggested (3, 7, 8). IgG autoantibodies from patients with bullous pemphigoid could bind epitopes localized on three synthetic peptides encompassing the C-terminal end of BPAgl cDNA (7). Using an approach similar to ours, the existence of two Ab binding sites on BPAgl (9) has been reported. The same group of investigators recently extended their results using the same Abs on deleted clones of a mouse partial cDNA sequence encoding the mouse equivalent of BPAgl. They were able to map the reactivity of one Ab (5E-HY-4B) within 114 amino acid residues in the C-terminal domain of BPAgl (10). Unfortunately, the other Ab (10D-HY-8B) did not show any reactivity. From two patients, we obtained three HumAbs specific for BPAgl. As these HumAbs do not cross-compete for binding to natural BPAgl with an antisem from a rabbit immunized with rBP55, and as they do not cross-compete with each other, it is likely that 1) these 3 HumAbs may recognize the non-C-terminal portion of BPAgl, although we cannot exclude the recognition of a conformational/carbohydrate determinant not expressed by the recombinant rBP55 polypeptide; and 2) there are at least four different epitopes on BPAgl.

First group: R1 Second group: R2

Third group: R3

Anti-BPAgl HumAb: BP1, BP2, BP3.

BP patients sera: BPs

First group of reactivity: R1

Second group of reactivity: R2

Third group of reactivity: R3


