Definition of Bullous Pemphigoid Antibody Binding to Intracellular and Extracellular Antigen Associated With Hemidesmosomes

Diya F. Mutasim, M.D., Lynne H. Morrison, M.D., Yuzo Takahashi, M.D., Ph.D., Ramzy S. Labib, M.D., Ph.D., John Skouge, M.D., Luis A. Diaz, M.D., and Grant J. Anhalt, M.D.

Immunodermatology Unit, Department of Dermatology, The Johns Hopkins University School of Medicine, Baltimore, Maryland, U.S.A.

Bullous pemphigoid (BP) antibodies are deposited predominantly in the lamina lucida in vivo; however, circulating BP antibodies bind in vitro to the cytoplasmic plaque of basal cell hemidesmosomes. We examined the ability of IgG in nine BP sera to bind to intracellular or extracellular antigen.

On skin cryosections, indirect IF showed IgG bound to basement membrane zone (BMZ) and indirect ImmunoEM confirmed intracellular binding on the cytoplasmic plaque of hemidesmosomes.

In contrast, when normal skin was exposed to BP serum in organ culture, direct IF showed fainter linear deposition of IgG along the BMZ, and direct ImmunoEM demonstrated extracellular IgG binding in the lamina lucida, predominantly beneath hemidesmosomes.

Four of nine sera showed complement fixation on indirect IF samples (IgG bound to intracellular antigen) and three showed complement fixation on direct IF specimens (IgG bound to extracellular antigen). Three of the nine sera contained complement fixing antibodies detectable only in antibody populations specific for intracellular or extracellular antigen.

Western immunoblots showed that five of nine sera recognized a 240-kD protein and four of nine recognized a 180-kD protein. There was no correlation between the presence (or absence) of either band and the detection of complement fixing antibodies specific for intracellular or extracellular antigen.

BP autoantibodies bind both intracellular and extracellular antigen, and IgG binding exclusively to extracellular antigen that mimics the in vivo situation can be detected by using organ culture. Complement fixation may be restricted to antibodies specific for intracellular or extracellular antigen. These findings underscore the complexity of the autoantibody-antigen system in BP and have implications regarding the proposed pathogenicity of the autoantibodies. J Invest Dermatol 92:225–230

Bullous Pemphigoid (BP) is a chronic bullous dermatosis characterized by dermal-epidermal separation and IgG autoantibodies (BP, AB) against the basement membrane zone (BMZ). These antibodies can be detected both in lesional skin as well as in the patients' sera by immunofluorescence (IF) techniques. Studies using direct immunoelectron microscopy (ImmunoEM) on perillesional skin have shown the tissue bound BP AB to be located in the lamina lucida of the BMZ [1–3]. However, we, as well as others, have shown that by indirect ImmunoEM, the circulating BP AB detected in vitro are directed against the epidermal basal cell hemidesmosomes (HD) [4–8].

In this study we show that BP sera contain antibodies that can bind both to intracellular and extracellular (lamina lucida) antigens associated with the hemidesmosome. The detection of either of the antibody systems depends on the procedure used. In addition, we show that at least some BP sera the two antibody systems may be distinctive with regard to their complement fixation (CF) properties.

MATERIAL AND METHODS

To differentiate binding of BP antibodies to antigens within the cell or in the extracellular domain of the basal cell, skin was exposed to BP serum under two conditions. 1) Viable skin was placed in organ culture with BP serum incorporated in tissue culture medium. Because the cells would be viable, and the plasma membrane of the basal cell intact, antibody binding could occur only to extracellular antigen. 2) Alternatively, frozen sections were exposed to the BP serum, and because of the freezing and sectioning, both intra- and extracellular antigens would be able to bind antibody.

Materials Human skin was obtained from fresh surgical specimens. To eliminate variability due to possible regional variations in expression of BP antigen, only specimens from the head and neck

Abbreviations:
AB: Antibody
BMZ: Basement membrane zone
BP: Bullous pemphigoid
CF: Complement fixation
DAB: Diaminobenzidine
EDTA: Ethylenediaminetetraacetic acid
FITC: Fluorescein isothiocyanate
HD: Hemidesmosome
IF: Immunofluorescence
ImmunoEM: Immunoelectron Microscopy
M199: Medium 199
MEM: Minimal essential medium
NHS: Normal human serum
PBS: Phosphate buffered saline

Manuscript received March 16, 1988; accepted for publication August 17, 1988. This work was supported in part by U.S. Public Health Service Grants ROI-AR32599, ROI-AR32081, ROI-AR32490, KO4-AR01686, and T32-AR07324 from the National Institutes of Health, and by a grant from the Dermatology Foundation. Reprint requests to: Grant J. Anhalt, M.D., Department of Dermatology, The Johns Hopkins University, 600 N. Wolfe Street, Baltimore, MD 21205

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area of adults were used. In addition, individual skin specimens were divided into small enough pieces so that all manipulations (direct and indirect IF and ImmunoeM) could be compared on a single specimen. Specimens were dermatomed to 1 mm thickness and transferred into tissue culture medium. BP sera were obtained from nine patients with the typical clinical, pathological, and immunologic features of the disease. The antibody titer of these sera on monkey esophagus ranged from 1:320 to 1:2560. Normal human serum (NHS) and freshly frozen serum (the source of human complement) were obtained from a volunteer. Medium 199 (M199) was obtained from Flow Laboratories (McLean, VA), and Dulbecco’s Minimal Essential Medium (MEM) was obtained from Hazeltin (Lenexa, KS). Peroxidase labeled and fluorescein isothiocyanate (FITC) labeled antihuman IgG and antihuman C3 were all obtained from Cappel-Worthington (Cochranville, PA).

**Immunofluorescence Studies**

**Direct IF: IgG Binding:** Individual 2–4 mm pieces of dermatomed viable human skin were incubated with each of the nine sera and one control NHS at a 1:10 dilution in Dulbecco’s MEM supplemented with 13% fetal calf serum, at 4°C for 24 h. Skin was then washed in culture medium for 2–3 h, frozen, and 4 μm cryosections were obtained. To detect IgG binding, sections were treated with FITC antihuman IgG and washed in phosphate buffered saline (PBS).

**Indirect IF: IgG Binding:** Four-micron cryosections of normal human skin were incubated in each of nine BP sera or control NHS at 1:20 dilution in PBS for 30 min, washed in PBS, then incubated in FITC antihuman IgG for 30 min and washed.

**Direct IF: Complement Fixation:** To determine CF of the bound antibody, cryosections from the organ culture skin were first treated with fresh NHS (as a complement source) at 1:10 dilution in M199 for 30 min, washed in PBS, treated with FITC antihuman C3, and washed in PBS.

**Indirect IF: Complement Fixation:** For in vitro CF assays, cryosections of normal human skin were incubated with BP serum or normal human serum for 30 min, washed in PBS, then incubated with fresh NHS at 1:10 dilution in M199 at 37°C for 30 min, washed, and then treated with FITC antihuman C3.

All sera were tested three to four times by two “blinded” observers, and the intensity of positive reactions was graded on a scale of Negative to 4+. All IF slides were read with a Zeiss fluorescent microscope equipped with epifluorescence.

**Immunoelectron Microscopy**

**Direct ImmunoEM: IgG Binding:** For direct ImmunoEM, viable skin was incubated under organ culture conditions with the test or control serum, washed with M-199 for 3 h at 4°C, cryosectioned, and then incubated with peroxidase-conjugated antihuman IgG (Cappel-Worthington).

**Indirect ImmunoEM: IgG Binding:** For indirect ImmunoEM, cryosections of normal skin were incubated sequentially with the BP or control sera and then peroxidase conjugated antihuman IgG.

**Direct ImmunoEM: Complement Fixation:** For complement fixation on direct ImmunoEM specimens, after organ culture, cryosections were incubated with fresh human serum and then peroxidase-conjugated antihuman C3 (Cappel-Worthington).

**Indirect ImmunoEM: Complement Fixation:** For in vitro complement fixation on indirect ImmunoEM specimens, cryosections were incubated sequentially with BP or control sera, fresh human serum, and peroxidase conjugated antihuman C3.

After these incubations, all sections were treated with diaminobenzidine (DAB) (Sigma, St. Louis, MO) for 5–10 min, fixed in 1% glutaraldehyde for 1 h, washed in PBS, post-fixed in 1% osmium tetroxide for 1 h, dehydrated in a series of ascending alcohol concentrations, and embedded in British Araldite. Sections were examined without counterstaining for better visualization of the reaction product.

**Immunoblots** All sera were tested for detection of BP antigen by the method of Labib et al [9]. Briefly, human skin was obtained from surgical specimens, and epidermis was split from dermis by overnight incubation in PBS with 2 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM phenylmethylsulfonylfluoride (PMSF) at room temperature. Epidermis was extracted in 10 mM Tris, pH 8.0, with 1% sodium dodeyl sulfate, 2 mM PMSF, 2 mM EDTA, b-mercaptoethanol, and six bacterial proteinase inhibitors [9]. The extract was electrophoresed in a 7% polyacrylamide gel and electrophoretically transferred to nitrocellulose paper by the method of Towbin et al [10]. Individual lanes were incubated sequentially with a 1:100 dilution of patient serum, a 1:1000 dilution of biotinylated goat antihuman IgG, and a 1:10000 dilution of peroxidase labeled strepavidin (all from Southern Biotechnologies Ltd., Birmingham, AL). Reaction product was developed using DAB. All sera were blotted on three occasions.

**Cell Viability** To determine viability of basal cells obtained after 24 h of incubation under organ culture conditions, portions of the organ culture skin were treated with trypsin 0.25% for 30 min at 37°C, and dissociated basal cells were obtained by discontinuous ficoll gradient centrifugation [5]. Viability was determined by Trypan blue exclusion. Cells were also air dried onto glass slides and either treated with FITC anti-IgG alone or sequentially with BP serum then FITC anti-IgG.

**RESULTS**

**Immunofluorescent Studies**

**Direct IF: IgG Binding:** In specimens exposed to antibody under organ culture conditions, by 6 h there was evidence of BMZ staining only at the periphery of the specimen. After 24 h of incubation, all BP sera showed BMZ IgG binding in a continuous linear pattern with three notable features. First, the intensity of IgG BMZ staining was much weaker than that observed when specimens were tested by indirect IF. Second, at the periphery, and intermittently throughout the BMZ of the specimen, there were individual cells or groups of basal cells in which the staining was much more intense (Figs 1 and 2). The intensity of this focal fluorescence was equivalent to the intensity of the BMZ staining that was seen by indirect IF (Fig 3). When direct IF sections from organ culture that demonstrated the weak BMZ BMZ binding were reincubated with the respective BP serum and treated for a second time with FITC anti-human IgG, the intensity of BMZ staining increased significantly, the BMZ staining became uniform, and appeared identical to that seen in indirect IF specimens. Third, there was striking variation in the overall intensity of BMZ staining among the different BP sera.

**Indirect IF: IgG Binding:** When using cyosections of skin, all nine BP sera contained autoantibodies that bound along the BMZ in the expected linear pattern [5] with almost identical intensity (Fig 3).

**Direct IF: Complement Fixation by Bound Antibody:** Positive specimens showed linear/granular fluorescence along the BMZ. Three sera contained antibodies capable of complement fixation by the direct technique but six did not (Table I). Of the three sera containing complement fixing antibody, two also showed complement fixation by indirect IF but one did not. Similarly, two sera that were able to fix complement by indirect IF were not able to fix complement using the direct IF technique (Figs 4 and 5). Therefore, three sera contained complement fixing antibodies that were restricted to one or the other population of autoantibodies, detected by direct and indirect CF techniques.

**Indirect IF: Complement Fixation by Bound Antibody:** Using routine indirect IF, four of the nine BP sera fixed complement to the BMZ in a finely granular pattern, as expected. The bound antibody of five sera was not complement fixing.

Individual sera produced identical immunofluorescent findings (for both IgG binding and complement fixation) in multiple specimens, and the interpretation was consistent between blinded observers.
Figures 1-3. 1 and 2: Direct immunofluorescence for IgG: viable skin explants were incubated with a Bullous pemphigoid (BP) serum (serum MA, Table I) in organ culture medium for 24 h, washed, cryosectioned, and then treated with fluorescein labeled antihuman IgG. Throughout most of the dermal-epidermal junction there is faint linear deposition of IgG, demonstrating binding of antibody to extracellular BP antigen (small arrowheads). In scattered foci there is intense fluorescence at the base of single basal cells or clusters of basal cells (open arrows). This focal bright fluorescence is presumed to be due to penetration of the antibody into the cytoplasm of dead cells, where it can bind intracellular antigen. This demonstrates the relative intensity produced by binding to extracellular or intracellular antigen within a single specimen using a single serum. 3: Indirect immunofluorescence for IgG: normal human skin cryosections were incubated with the same BP serum used in 1 and 2 for 30 min, followed by fluorescein labeled antihuman IgG. A bright, continuous linear band of fluorescence is seen at the dermal-epidermal junction, and this represents antibody binding to both intracellular and extracellular antigen. The intensity of antibody binding is much greater than that observed in the majority of the organ culture specimens shown in 1 and 2, but is roughly equivalent to that seen in the presumed dead cells.

Table I. Summary of the Characteristics of the Nine Bullous Pemphigoid (BP) Sera Studied

<table>
<thead>
<tr>
<th>Patient</th>
<th>IF*</th>
<th>DIF*</th>
<th>ICIF*</th>
<th>DCIF*</th>
<th>Immunoblot*</th>
</tr>
</thead>
<tbody>
<tr>
<td>RU</td>
<td>4+</td>
<td>3+</td>
<td>2-3+</td>
<td>3+</td>
<td>240,180 KD</td>
</tr>
<tr>
<td>TA</td>
<td>4+</td>
<td>3+</td>
<td>3+</td>
<td>3+</td>
<td>180 KD</td>
</tr>
<tr>
<td>MA</td>
<td>3+</td>
<td>1+</td>
<td>NEG.</td>
<td>NEG.</td>
<td>240 KD</td>
</tr>
<tr>
<td>SO</td>
<td>3+</td>
<td>1+</td>
<td>NEG.</td>
<td>NEG.</td>
<td>240 KD</td>
</tr>
<tr>
<td>YO</td>
<td>3+</td>
<td>2+</td>
<td>NEG.</td>
<td>NEG.</td>
<td>240 KD</td>
</tr>
<tr>
<td>MA</td>
<td>3+</td>
<td>2+</td>
<td>NEG.</td>
<td>NEG.</td>
<td>240 KD</td>
</tr>
<tr>
<td>SM</td>
<td>3+</td>
<td>2+</td>
<td>NEG.</td>
<td>2-3+</td>
<td>240 KD</td>
</tr>
<tr>
<td>LA</td>
<td>3+</td>
<td>1+</td>
<td>1-2+</td>
<td>NEG.</td>
<td>180 KD</td>
</tr>
<tr>
<td>TH</td>
<td>3+</td>
<td>2+</td>
<td>3-4+</td>
<td>NEG.</td>
<td>180 KD</td>
</tr>
</tbody>
</table>

* IF: indirect immunofluorescence on human skin. All sera had detectable BP antibodies, bound to the basement membrane zone (BMZ) and graded on a scale of 0-4+.
* DIF: direct immunofluorescence, on skin exposed to BP sera in organ culture so that only extracellular antigen is available to bind BP antibodies. All sera had BMZ antibodies under these conditions, but the binding was much weaker than with IF.
* ICIF: indirect complement immunofluorescence. Four sera had complement-fixing BMZ antibodies, bound to the BMZ by indirect IF.
* DCIF: direct complement IF. This shows the complement-fixing activity of BMZ antibodies bound under organ culture conditions (extracellular antigen only). In three sera, complement fixation was restricted to antibodies bound in one technique, but not the other (ICIF vs DCIF).
* Immunoblotting: All sera were tested by Western immunoblotting for the presence of antibodies specific for previously reported BP antigens (Mr. 240 kD and 180 kD). These results are shown in the final column of the Table.

Figures 4 and 5. 4: Direct immunofluorescence for C3. Viable skin explants were incubated with BP serum LA (Table I) in organ culture medium for 24 h, washed, cryosectioned, and treated with fresh normal human serum (as a complement source) then fluorescein labeled antihuman C3. Although this patient had detectable IgG binding along the dermal-epidermal junction, no complement fixation by these antibodies was detected (BMZ outlined by arrowheads). This apparently shows that the antibodies binding to extracellular BP antigen were not complement fixing. 5: Indirect immunofluorescence for C3. Normal human skin cryosections were incubated with BP serum LA (the same serum used in 4), followed by fresh normal human serum (complement source) then fluorescein labeled antihuman C3. Dense granular fluorescence is seen at the dermal-epidermal junction (open arrow). This shows that the complement fixing properties of the BP antibodies in this patient's serum are restricted to those that bind intracellular antigen only.
ImmunoEM Studies

Direct ImmunoEM: IgG Binding: Direct ImmunoEM for IgG binding under organ culture conditions showed immunoreactants located only in the upper lamina lucida, with the majority of the deposits located in the lamina lucida immediately beneath hemidesmosomes (Fig 6). No deposits were seen in desmosomes. At the periphery of the specimens, and intermittently in individual basal cells or groups of basal cells, immunoreactants were also seen on the cytoplasmic plaque of hemidesmosomes (similar to that observed in indirect ImmunoEM specimens). There was no IgG deposition detected in specimens treated with NHS.

Indirect ImmunoEM: IgG Binding: Indirect ImmunoEM performed on specimens exposed to antibody after cryosectioning showed IgG binding in the area of the hemidesmosomes. The immunoreactants were deposited in the form of clumps which appeared to extend from the external face of the basal cell membrane into the area of the cytoplasmic plaque of the hemidesmosome (Fig 7). Precise definition of the limit of the immunoreactants was difficult because the specimens were not counterstained. NHS did not contain any IgG capable of binding within the BMZ.

Direct ImmunoEM: Complement Fixation by Bound Antibody: By ImmunoEM, complement deposition was found within the lamina lucida and adjacent to the plasma membrane of the basal cells (Figs 8 and 9). Complement was more diffusely deposited than IgG within the lamina lucida, and there was no apparent restriction to the area underlying hemidesmosomes. No complement deposition was seen in specimens incubated with NHS.

Indirect ImmunoEM: Complement Fixation by Bound Antibody: By indirect ImmunoEM, the complement was found to be deposited in the cytoplasmic side of the plasma membrane of the basal cell and overlying the plaque region of the hemidesmosomes (Fig 10). The distribution of complement deposition coincided quite closely with the observed distribution of bound IgG in indirect specimens. There was no apparent deposition within the lamina lucida. No complement deposition was detected in specimens treated with normal human sera.

Immunoblotting Eight of the nine BP sera detected at least one of the antigens previously reported to be identified by BP autoantibodies in blot analysis (Table I). Five of the nine recognized a 240-kD antigen, while four of the sera recognized a 180-kD antigen. One serum recognized both antigens. No lower M.W. bands were seen. There was an observed association between the presence of complement fixing antibodies to the intracellular antigen (indirect complement fixation IF) and the detection of the 180-kD band by blotting, but this was not significant, given the small sample size. One serum did not recognize any specific band by Western blot.

Cell Viability At 24 h, basal cells had viability of 90%. When they were treated with FITC anti-IgG none showed any staining. However, when treated with BP serum then FITC anti-IgG, all basal cells showed the classical polar staining (not shown).

DISCUSSION

Originally, ImmunoEM studies that attempted to localize the antibody binding site in BP were performed on lesional and perilesional skin from patients with the disease. These studies showed that the BP AB were bound predominantly within the lamina lucida, but Holubar et al [1] and Schmidt-Ullrich et al [2] reported additional binding of IgG on hemidesmosomes. Trost et al [3] also detected complement deposition both in a thin band within the lamina lucida, and in clumps overlying hemidesmosomes. The observation of BP AB binding within cells was not pursued for a long time. Recently, however, when testing BP sera by indirect ImmunoEM, we as well as other investigators have shown that in vitro the circulating BP AB bind within the cytoplasm of the basal cell in the region of the HD plaque [5–8]. Yamazaki and Nishikawa also reported that when BP sera were tested in indirect ImmunoEM, IgG binding was present within the cell in individual sera, but complement fixation by those sera occasionally occurred in the lamina lucida as well [11]. The significance of this observation was not clear. The purpose of our investigation was to study the discrepancy between the reported ultrastructural binding sites of BP autoantibodies. By exposing viable skin to BP serum in organ culture, we could mimic the antibody binding that occurs in vivo and contrast our findings to those observed when skin is first sectioned and exposed to BP serum in vitro. We selected nine BP sera known to have circulating BP antibodies. When indirect IF and indirect ImmunoEM were performed using individual sera, the ultrastructural studies showed that the BMZ IF staining resulted from binding of BP AB to basal cell HD, as previously reported [4–7]. In the direct technique, when the tissue was exposed to BP sera under organ culture conditions, the results were quite different. First, the intensity of BMZ staining by IF was much weaker and more variable than that detected by indirect IF on skin sections using the same serum. Second, at the edge of
the specimen, and in multiple foci throughout the BMZ, the intensity of staining was much stronger, with an intensity similar to that detected by indirect IF. Direct ImmunoEM, performed on these specimens, showed the BP antibody bound within the lamina lucida in most of the specimen, with occasional cell or group of cells showing antibody bound within the cell in the hemidesmosome.

Our interpretation of the above observations is as follows. In the direct technique the plasma membrane of the cell is intact and BP AB can only bind the accessible pool of BP antigen located in the lamina lucida. Our viability studies showed that about 10% of basal cells in these cultures were dead. The focal brightly staining cells seen by IF likely represent these dead cells. Because the plasma membrane of these cells is no longer intact, the staining is more intense due to binding of antibody within the hemidesmosome. The observation that the BMZ staining detected by direct IF is weaker than that detected by indirect IF using the same serum may imply a quantitative difference between the amount of antigen present in the lamina lucida and that in the HD. Alternatively, this may represent a difference in antigen-antibody affinity between the two pools of antigen. Finally, there was a significant variability in the intensity of the direct IF BMZ staining among the different sera, despite the fact that all sera had consistently very strong BMZ staining by indirect IF. This implies that there is a greater variability in the titer of BP antibodies against the lamina lucida antigen in the individual sera.

This finding raises some important issues. The majority of EM studies performed on perilesional skin from patients with BP showed IgG binding only in the lamina lucida. It may be that the antibody that binds the lamina lucida antigen is the pathogenic antibody, and the antibodies that bind the HD antigen have no role in blister formation in vivo. The difference in the intensity of BP AB binding to intracellular or extracellular antigens detected in the serum may reflect a variability in the amount of the two BP AB systems in different stages of the disease. It is conceivable that in active stages of BP, the lamina lucida BP AB may be bound avidly to the patient’s skin, thus the circulating lamina lucida BP AB may be present in only small amounts. Indeed, the two BP sera which had the weakest lamina lucida binding were from patients with active disease.

The significance of the HD directed BP antibody is not clear at present. If it turns out to be unrelated to the pathogenesis of blister formation in BP, it would explain why, in most studies, there is no correlation between disease activity and “antibody titer” and why in some patients in remission with negative direct IF, the circulating BP antibody titer may be high [12–14]. The study should show that the intensity of the BMZ fluorescence produced by BP AB that bind the HD antigen is much greater than that produced by BP AB that bind the lamina lucida antigen. The “antibody titer” as typically detected by indirect IF is primarily a reflection of the circulating BP antibody directed against HD.

The results of the CF assays by both the direct and indirect procedures were also intriguing. As expected, about half of the sera were positive by the classical indirect CF assay. However, by direct CF, some “complement fixing” sera were negative and one “non-complement fixing” serum was positive. The significance of this lack of correlation between the CF properties of the two BP antibody systems is unknown at present, but it strengthens our hypothesis that the two antibody systems are distinctive. Until now, detecting the “complement fixing” properties of BP sera has been done by the indirect assay. When studying pathogenetic mechanisms of BP, it may be important to examine the CF titer of the lamina lucida BP AB system because circumstantial evidence suggests that this system is involved in lesion induction [15–19]. This can be done by measuring CF titers of BP AB that bind only under organ culture conditions.

It is becoming increasingly apparent that the characterization of BP autoantibodies and the antigen(s) to which they bind is a complex task. For example, Stanley and co-workers have established that circulating antibodies from BP patients identify a 220–240-kD molecule both by immunoprecipitation and by immunoblotting.
However, Labib et al [9], using Western immunoblots, found that ~50% of BP sera recognize a 240-kD protein (that is likely the same antigen originally detected by Stanley et al), however, about one-third of BP sera recognized a 180-kD epidermal antigen. Sometimes this was the only antigen detected, but in some sera both antigens were detected. The ultrastructural distribution of each of these antigens is not yet defined, but Jones has recently described a 180-kD protein component of the basal cell HD that may be the same antigen detected by these BP sera [22]. Our immunoblotting data did not show any correlation between the presence of antibodies to one or the other band and other characteristics, such as complement fixation restricted to AB bound to intracellular or extracellular antigen.

This study also provides some evidence that the BP antigen(s) may be transmembrane proteins. If this is true, they may represent an important link between the extracellular matrix and the hemidesmosome-cytoskeleton. This would imply that they may have important functions in normal cell-matrix adhesion as has been postulated in the past [23].

In conclusion, we have shown that BP sera have circulating autoantibodies capable of binding to both intracellular and extracellular antigen. The detection of antibody binding to the two pools of antigen is dependent on the technique employed. The relative significance of the autoantibodies that are specific for each of the antigen pools, and the significance of their occasionally mismatching complement fixation properties in the pathogenesis of BP, will be the subject of further studies.

REFERENCES