Human Autoantibodies against the 230-kD Bullous Pemphigoid Antigen (BPAG1) Bind Only to the Intracellular Domain of the Hemidesmosome, whereas Those against the 180-kD Bullous Pemphigoid Antigen (BPAG2) Bind along the Plasma Membrane of the Hemidesmosome in Normal Human and Swine Skin

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Abstract

Bullous pemphigoid (BP) is a blistering skin disease in which autoantibodies develop to hemidesmosomal components of the epidermal basement membrane zone, including two major antigenic proteins of the 230-kD antigen (BPAG1) and the 180-kD antigen (BPAG2). The present study demonstrated the precise ultrastructural localization of the epitopes for autoantibodies against BPAG1 and BPAG2 in normal skin. Autoantibodies against either BPAG1 or BPAG2 were affinity-purified using nitrocellulose membrane, which was blotted with SDS-PAGE-fractionated antigens from human epidermal extract as the immunoabsorbent. Postembedding, immunogold electron microscopy was performed after skin was processed by rapid freezing and freeze substitution fixation without chemical fixatives. Purified autoantibodies against BPAG1 bound only to the intracellular domain of the hemidesmosome, and 80% of the gold labeling was within 40–140 nm from the plasma membrane (mean distance 91 nm inside). In contrast, the autoantibodies against BPAG2 bound along the plasma membrane of the hemidesmosome, and 80% of the gold labeling was within 10 nm outside to 50 nm inside the cells (mean distance 12 nm inside). These results suggest that the autoantibodies against BPAG1 and BPAG2 react with the epitopes localizing in distinct regions of the hemidesmosome complex, and may play different roles in the blister formation in patients with BP. (*J. Clin. Invest.* 1993. 91:1608–1615.) Key words: autoimmune disease • basement membrane • dermoepidermal adhesion • immunoblot • postembedding immunoelectron microscopy

Introduction

Bullous pemphigoid (BP)[1] is a life-threatening blistering skin disease in which the presence of autoantibodies to the basement membrane zone (BMZ) of the skin (1) is a hallmark in its diagnosis. The binding of these autoantibodies to antigens located in the BMZ plays an important role in the pathogenesis of blister formation in BP (2–4). BP antigens are normal components of the hemidesmosome complex of the BMZ, which are thought to be important not only in the pathogenesis of BP but also in dermoepidermal adhesion. Since Diaz and his coworkers initially reported small molecular weight antigenic proteins recognized by BP sera (5, 6), considerable efforts have been spent in characterizing the BP antigen (7–20). Recently, a 220–240-kD protein (230-kD BP antigen, BPAG1) and a 180–160-kD protein (180-kD BP antigen, BPAG2) have been identified as two major BP antigens by immunoprecipitation (7) and Western immunoblotting (10, 12, 13, 14). More recently, the cDNA for BPAG1 (15–18) and the cDNA for BPAG2 (19–21) have been isolated and characterized.

The ultrastructural localization of BP antigens has been studied by various immunoelectron microscopic techniques. Preembedding immunoelectron microscopic studies, mainly using horseradish peroxidase as a probe, had revealed that BP antigens are associated with hemidesmosome of the basal keratinocytes (11, 22–27). However, the deposition of the diaminobenzidine products of a peroxidase probe is too extensive to determine the fine localization of these antigens, and also tends to obscure the underlying ultrastructure. Immunogold probes have various advantages over the immunoperoxidase technique and can be used for preembedding immunoelectron microscopy (28). However, gold-conjugated probes have the inevitable disadvantage of limited permeation into the tissue sample. Consequently, intracellular and extracellular antigens cannot be studied equally well by preembedding immunogold electron microscopy.

The actual binding sites of autoantibodies against BPAG1 and BPAG2 are thought to be important with regard to the mechanism of blister formation in BP. The different cDNA sequences of BPAG1 and BPAG2 confirmed that these BP antigens are different gene products. However, a previous preembedding immunoperoxidase electron microscopic study showed that the distribution of BPAG1 and BPAG2 on the hemidesmosome complex was identical (29). A preembedding immunogold electron microscopic study also showed similar binding sites on the plaque of hemidesmosome for both affinity-purified autoantibodies against BPAG1 and those against BPAG2 (30).

We have previously established a postembedding immunogold electron microscopy method that utilizes rapid freezing and freeze substitution fixation without chemical fixatives, which enables excellent preservation of both the ultrastructure and antigenicity of skin tissue. Using this technique, we have recently reported that autoantibodies from two BP patients bound to both the intracellular and extracellular domains of

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1. *Abbreviations used in this paper:* BMZ, basement membrane zone; BP, bullous pemphigoid; BPAG1, 230-kD bullous pemphigoid antigen; BPAG2, 180-kD bullous pemphigoid antigen; TBS, Tris-HCl-buffered saline.

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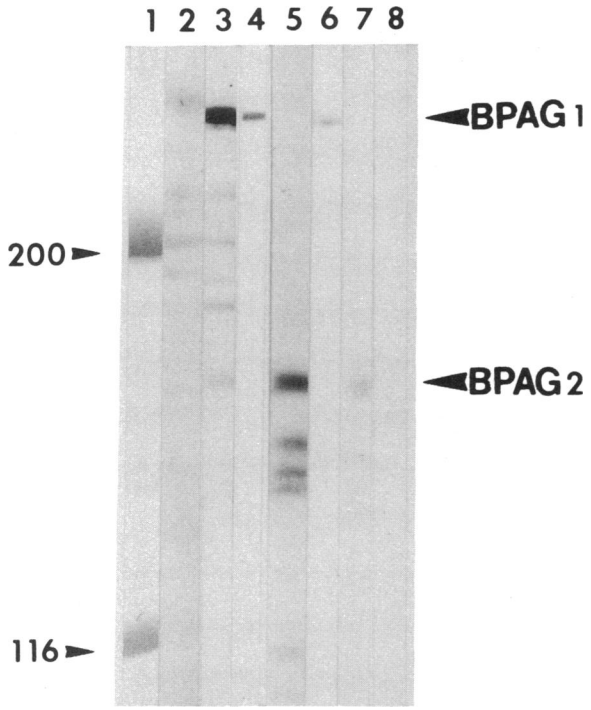


Figure 1. Western immunoblotting of 20 mM EDTA-separated human epidermal extract with whole BP sera and affinity-purified autoantibodies. Lane *1*, molecular weight markers; lane *2*, blot stained with amido black; lane *3*, binding of BP serum reacting with both BPAG1 and BPAG2. lane *4*, binding of BPAG1-specific BP serum; lane *5*, binding of BPAG2-specific BP serum that recognizes BPAG2 and its breakdown products; lane *6*, binding of affinity-purified anti-BPAG1 IgG; lane *7*, binding of affinity-purified anti-BPAG2 IgG; lane *8*, control antibodies eluted from the irrelevant strip of blot.

the hemidesmosome, but mainly to the intracellular domain (31). More recently, it has been shown that the carboxy terminus of BPAG1 is localized only in the intracellular domain of the hemidesmosome (32).

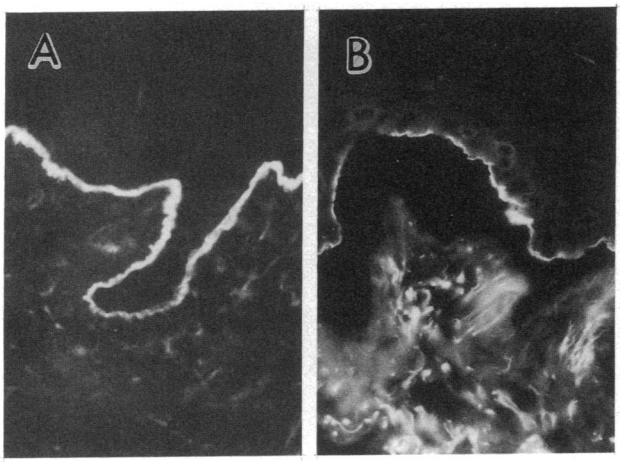


Figure 2. Indirect immunofluorescence with affinity-purified antibodies. (*A*) Affinity-purified anti-BPAG1 IgG showed a linear staining along the BMZ of normal human skin. (*B*) Affinity-purified anti-BPAG1 IgG bound only to the roof of the 1 M NaCl-separated normal human skin. Affinity-purified anti-BPAG2 IgG showed an identical pattern. Control serum and the control antibodies did not show any specific binding (not shown).

The purpose of this study was to demonstrate the precise ultrastructural localization of the epitopes on BPAG1 and BPAG2, where patient autoantibodies actually bind in normal human skin and normal swine skin. For this, we employed postembedding immunogold electron microscopic technique using rapid freezing and freeze substitution fixation in conjunction with affinity-purified BP autoantibodies. Here we report evidence that autoantibodies directed against BPAG1 only bind to the intracellular domain of the hemidesmosome, whereas those directed against BPAG2 bind along the plasma membrane of the hemidesmosome complex.

Methods

Sera. Sera obtained from three clinically typical BP patients were used in this study. Indirect immunofluorescence showed each serum had circulating anti-BMZ antibodies at a titer of 1:40 to 1:1,280. By Western immunoblotting (see below), the first serum (referred to as BPAG1-specific BP serum) reacted only with the 230-kD BP antigen (BPAG1), the second serum (referred to as BPAG2-specific BP serum) reacted only with the 180-kD BP antigen (BPAG2), and the third

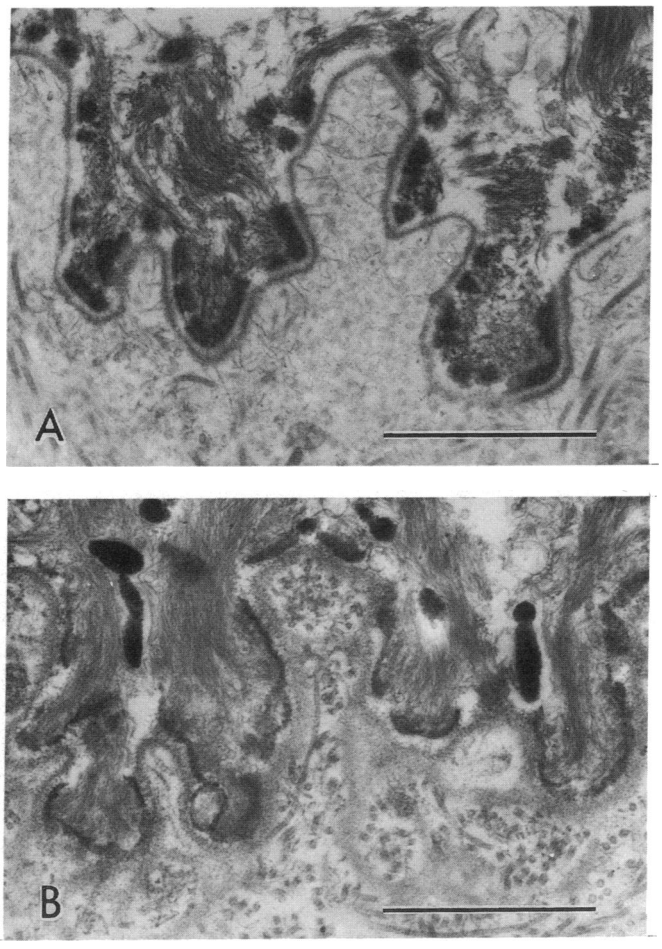
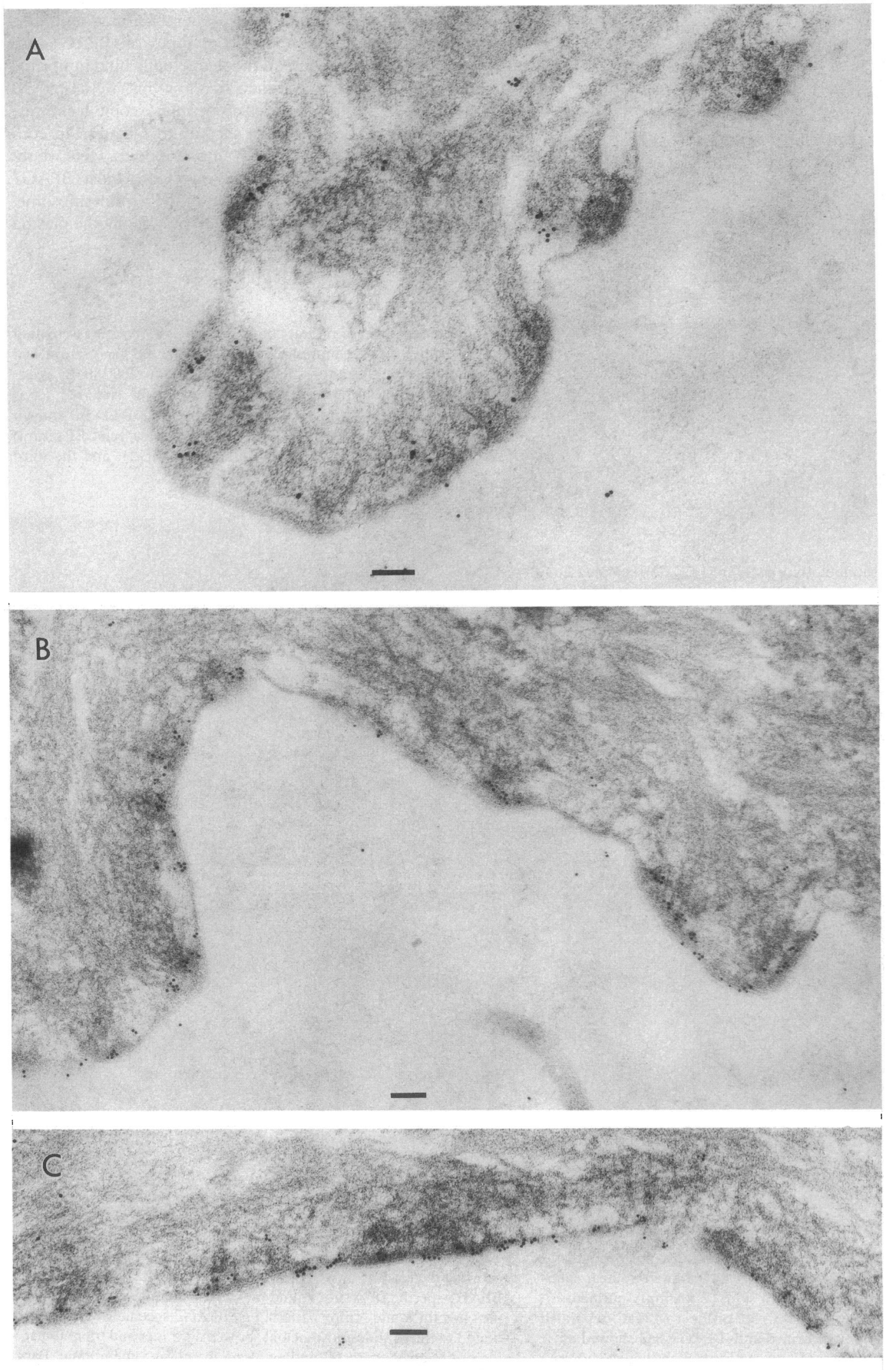


Figure 3. Preembedding immunoperoxidase electron micrographs showing the binding of whole BP sera to the normal human skin. (*A*) BPAG1-specific BP serum bound only to the hemidesmosome complex in a thick and lumpy pattern. (*B*) BPAG2-specific BP serum also bound to the hemidesmosome but showed a thinner and more linear discontinuous pattern of binding along the plasma membrane. Bars, 1 μm.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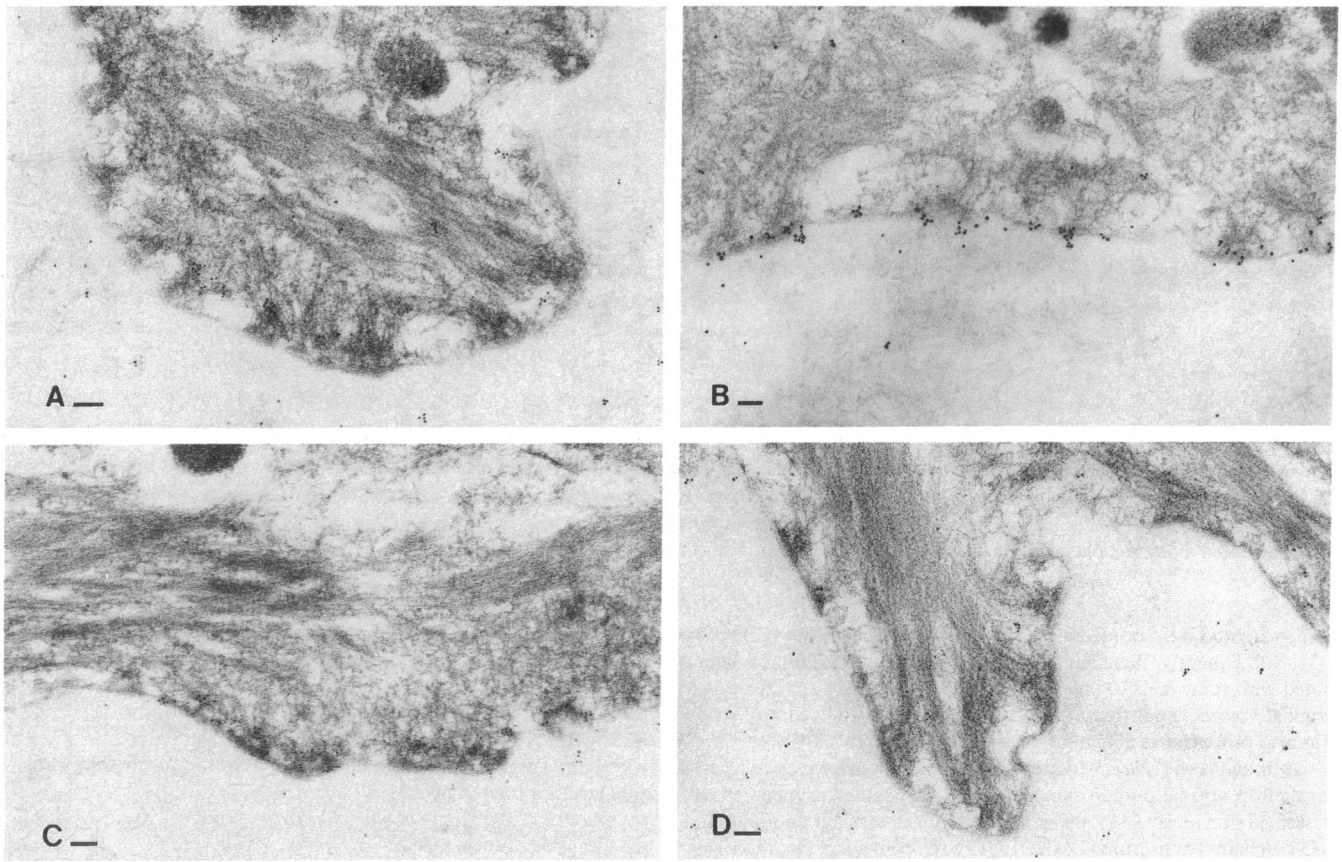

Figure 5. Postembedding immunogold electron micrographs of normal human skin. (*A*) Affinity-purified anti-BPAG1 IgG. Labeling located only in the intracellular portions of the hemidesmosome, away from the plasma membrane, mainly within 40–140 nm of the plasma membrane. (*B*) Affinity-purified anti-BPAG2 IgG. Labeling was distributed along the plasma membrane of the hemidesmosome within 50 nm intracellularly and 10 nm extracellularly. (*C*) BP serum reacting with both BPAG1 and BPAG2 (the antibody source serum of anti-BPAG1 and anti-BPAG2). Labeling located both along the plasma membrane and the intracellular portion of the hemidesmosome away from the plasma membrane. This distribution of labeling corresponded to a combination of the binding sites of affinity-purified anti-BPAG1 IgG and anti-BPAG2 IgG (not enhanced with silver). (*D*) The control antibodies showed no specific labeling. Bars, 0.1 μm.

serum reacted with both BPAG1 and BPAG2. One normal human serum was used as a control.

Antigen source. Antigen extraction for immunoblotting was performed by the method of Meyer et al. (33) with slight modifications. Briefly, a foreskin obtained at surgery was cut into strips and incubated in PBS containing 20 mM EDTA, 1 mM PMSF at 4°C for 24 h. After being separated from the dermis, the epidermis was homogenized in Tris-HCl–buffered saline (TBS), pH 7.6, supplemented with 1.5% SDS, 2 mM PMSF, 5% β-mercaptoethanol, 2 mM EDTA, 5 mg/liter each of leupeptin, antipain, chymostatin, and pepstatin A. After being boiled for 5 min and centrifuged at 4°C for 30 min at 15,000 rpm in microcentrifuge, the supernatant was recovered and stored at −80°C.

Western immunoblotting. Immunoblotting of EDTA-separated normal human epidermal extract was performed as described previously (13).

Affinity-purification of autoantibodies. Autoantibodies specific either to BPAG1 or to BPAG2 were affinity-purified from the serum reacting with both BPAG1 and BPAG2, by the methods described previously (34, 35). Proteins in epidermal extract were separated by SDS-PAGE of a 5% separating gel (36) and electrophoretically transferred to nitrocellulose sheets (13 × 10 cm) (37). After blocking with 3% BSA in TBS, three vertical strips were cut from the center and both sides of each sheet. They were processed for immunoblotting with BP serum reacting with both BPAG1 and BPAG2 in order to determine the location of the BP antigens. Two horizontal 4 mm-wide strips, containing BPAG1 and BPAG2, respectively, were cut from an unstained nitrocellulose sheet. As a control, another horizontal strip was also cut from the nitrocellulose sheet below the 180kD band. All the strips were incubated at 4°C overnight with shaking in the BP serum, thereby reacting with both BPAG1 and BPAG2 diluted 1:40 in TBS with 0.5% BSA and 0.05% Tween 20. The strips were washed four times with 0.05% Tween 20 in TBS, and then washed twice with distilled water. Specific autoantibodies bound to BPAG1 and BPAG2 were eluted at 37°C for 30 min with 20 mM sodium citrate (pH 3.2) containing 0.5% BSA and 0.05%

Figure 4. Postembedding immunogold electron micrographs showing the binding of whole BP sera to the normal human skin. (*A*) BPAG1-specific BP serum bound to the intracellular portion of the hemidesmosome where tonofilaments were inserted. (*B*) BPAG2-specific BP serum bound along the plasma membrane of the hemidesmosome complex. There was no labeling of the plasma membrane between hemidesmosomes. (*C*) Binding of BPAG2-specific BP serum along the plasma membrane was observed better when the plasma membrane was cut perpendicularly. Gold particles were distributed both intracellularly and extracellularly across the plasma membrane of the hemidesmosome complex. Bars, 0.1 μm.

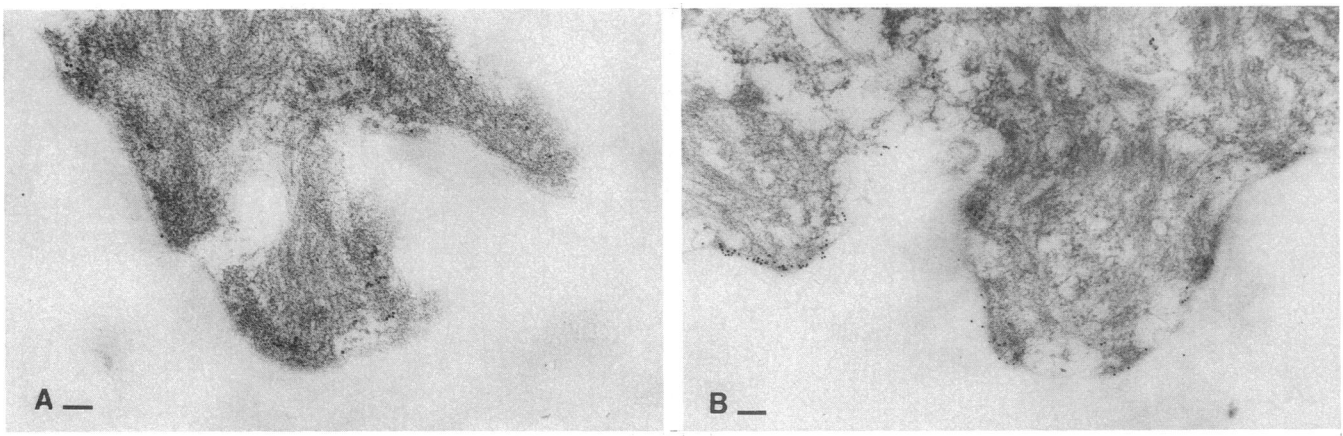

Figure 6. Postembedding immunogold electron micrographs of normal swine skin. The ultrastructure was almost the same as in human skin, but the nonspecific labeling decreased markedly. (*A*) Affinity-purified anti-BPAG1 IgG. Labeling located only in the intracellular portion of hemidesmosome away from the plasma membrane at the sire where tonofilaments were inserted. (*B*) Affinity-purified anti-BPAG2 IgG. Labeling was distributed along the plasma membrane of the hemidesmosome complex. Bars, 0.1 μm.

Tween 20, and were immediately neutralized with 2 M Tris-HCl (pH 7.5). Subsequently, these eluates were dialyzed against TBS, concentrated with centriprep 10 (Amicon Corp., Danvers, MA) to 2% of the original volume, and stored at 4°C. The eluate from the control strip was also processed as above for use as the control.

Immunofluorescence. Indirect immunofluorescence was performed on normal human skin and 1 M NaCl-split skin prepared as described previously (38), using FITC-conjugated rabbit anti-human IgG (γ-chains) antiserum (DAKOPATTS, Copenhagen, Denmark) as a secondary antibody.

Preembedding immunoperoxidase electron microscopy. Preembedding immunoperoxidase electron microscopy was performed as described previously (23, 27). Briefly, 6-μm cryostat sections of fresh normal human skin or 7-wk-old normal swine skin were incubated with 1:40 diluted BPAG1-specific BP serum or BPAG2 specific BP serum. After incubating with peroxidase-conjugated rabbit anti-human IgG (γ chains) antiserum (DAKOPATTS) diluted 1:40, the sections were prefixed with 2% glutaraldehyde, and reacted with 3,3′-diaminobenzidine. Then the sections were postfixed with 1% osmium tetroxide, dehydrated, and embedded in epon 812. Ultrathin sections were cut and observed under a transmission electron microscope (JEOL model 1200EX), with or without counterstaining with uranyl acetate and lead citrate.

Postembedding immunogold electron microscopy using cryofixation and freeze substitution. For postembedding immunogold electron microscopy, the skin tissue was processed by rapid freezing and freeze substitution fixation without chemical fixatives as described previously (31, 39). Briefly, fresh normal human skin and 7-wk-old normal swine skin were incubated at 4°C for 2 h with 15% glycerol in PBS for cryoprotection and then cut into small pieces (< 1 mm^3). The pieces were then rapidly frozen by plunging into liquid propane (−190°C), and cryosubstitution was performed by soaking in 100% methanol at −80°C for 48 h, followed by embedding in Lowicryl K11M (Chemische Werke Lowi, Waldkraiburg, FRG) at −60°C. The specimens were polymerized with UV radiation at −60°C for 72 h, and at room temperature for 72 h. Ultrathin sections were cut and incubated at room temperature for 1 h with 5% normal goat serum in washing buffer (0.8% BSA, 0.1% gelatin, and 2 mM NaN_3 in PBS, pH 7.4). The sections were incubated overnight at 4°C with BPAG1-specific BP serum, BPAG2-specific BP serum, BP serum reacting with both BPAG1 and BPAG2 (diluted 1:40), affinity-purified anti-BPAG1 IgG, affinity-purified anti-BPAG2 IgG, or control antibodies. The specimens were washed in the washing buffer, placed on a drop of 5 nm gold-labeled goat anti-human IgG (H + L) (Amersham International, Amersham, Buckinghamshire, UK) diluted 1:60 at room temperature for 2 h, and then subjected to further washing with the buffer and distilled water. To enhance 5-nm gold particle size for easier observation some sections were incubated at 4°C for 3 min with an immunogold silver enhancement solution (Amersham International), and then washed in distilled water as described previously (39). The specimens were counterstained with uranyl acetate and lead citrate, and observed under the transmission electron microscope.

Quantitative analysis of antigen distribution. To statistically evaluate the distribution of the binding sites for affinity-purified autoantibodies against BPAG1 and BPAG2, the distances from the outer surface of the plasma membrane to at least 500 randomly selected gold particles were measured on the electron micrographs. The result was analyzed statistically. The normality of the distribution was examined using the χ^2 test. The difference between BPAG1 and BPAG2 was determined by Wilcoxon's rank sum test.

Results

Western immunoblotting. With immunoblotting of the epidermal extract, BPAG1-specific BP serum and BPAG2-specific BP serum recognized exclusively a 230- and a 180-kD protein, respectively. Affinity-purified anti-BPAG1 IgG recognized only the 230-kD protein, but not the 180-kD protein. Affinity-purified anti-BPAG2 IgG recognized only the 180-kD protein and its breakdown proteins, but not the 230-kD band (Fig. 1). The control antibodies eluted from irrelevant nitrocellulose strips showed no specific reactivity.

Immunofluorescence. Both affinity-purified anti-BPAG1 IgG and affinity-purified anti-BPAG2 IgG showed linear staining to the BMZ of normal human skin. Both antibodies reacted only with the epidermal side of 1 M NaCl-separated normal human skin (Fig. 2).

Preembedding immunoperoxidase electron microscopy. Both BPAG1-specific BP serum and BPAG2-specific BP serum bound only to the hemidesmosome complex of the epidermal BMZ in both normal human skin (Fig. 3) and normal swine skin (not shown). BPAG1-specific BP serum showed the deposition with a thick and lumpy pattern at the intracytoplasmic attachment plaque (Fig. 3 *A*), while BPAG2-specific BP serum showed thinner and more linear deposit along the plasma membrane (Fig. 3 *B*). However, the relationship between the plasma membrane and the binding site of either antibody was

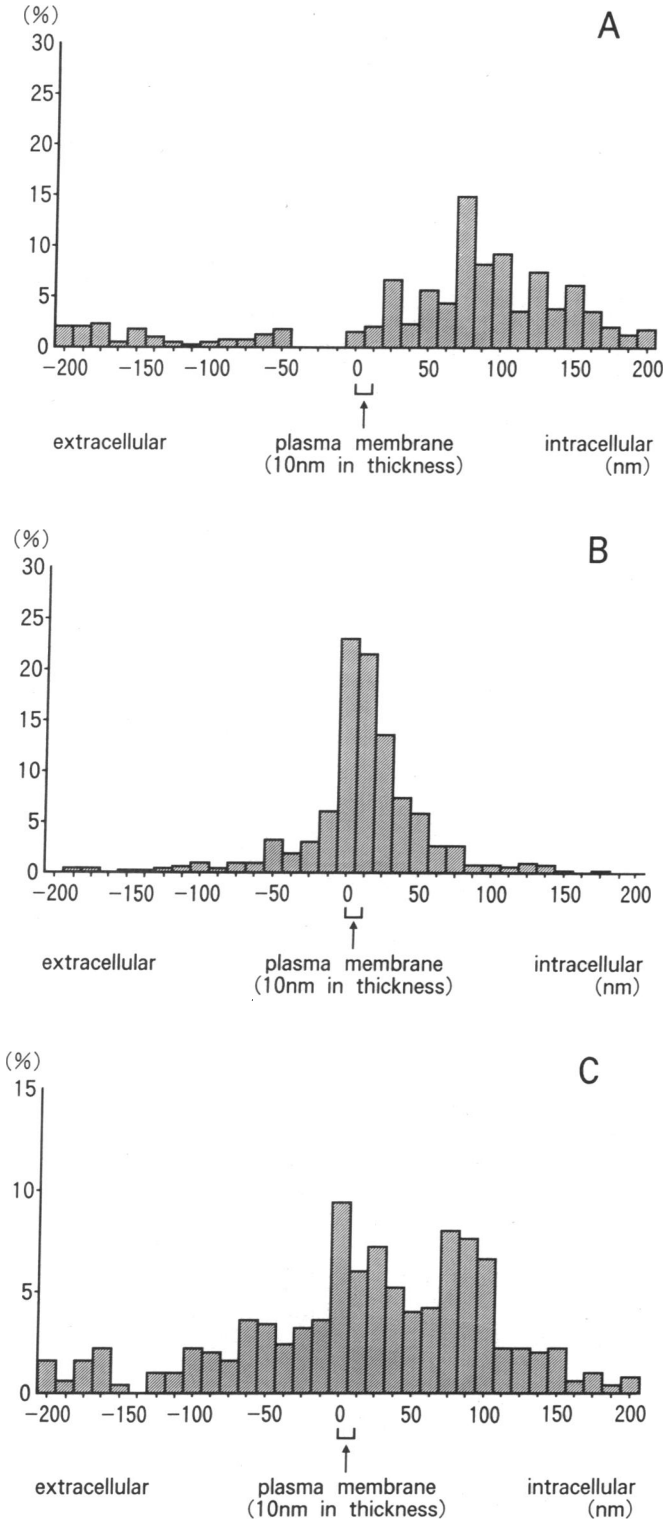

Figure 7. Distribution histograms of the distance between the plasma membrane and the binding sites. At least 500 gold particles were measured in each case. (*A*) The distribution of the binding sites of affinity-purified anti–BPAG1 IgG had a single peak at 75 nm. 80% of the labeling was within 40–140 nm of the plasma membrane. (*B*) The distribution of the binding sites of affinity-purified anti–BPAG2 IgG had a sharp single peak at 0 nm, a significantly different location from that of affinity-purified anti–BPAG1 IgG ($P < 0.0001$). The range of distribution was more narrow compared to that of affinity-purified anti–BPAG1 IgG. 80% of the labeling was found within 10 nm outside to 50 nm inside the cell. (*C*) The distribution of the binding sites of BP serum reacting with both BPAG1 and BPAG2 showed two peaks at 0 and 75 nm inside the plasma membrane. This distribution was a combination of binding sites of affinity-purified anti–BPAG1 IgG and BPAG2 IgG.

not clear because of the large diaminobenzidine products and the poor ultrastructural preservation of the plasma membrane.

Postembedding immunogold electron microscopy. The ultrastructure of the skin was well preserved. In both human and swine skin, the plasma membrane, hemidesmosomes, and tonofilaments were clearly observed, as reported previously (31, 39). Immunoelectron microscopic findings for the human and swine skin were substantially identical. The epitopes recognized by BPAG1-specific BP serum was only located on the intracellular portion of the hemidesmosome where the tonofilaments were inserted (Fig. 4 *A*). In contrast, the epitopes recognized by BPAG2 specific BP serum localized along the plasma membrane of the hemidesmosome complex (Fig. 4 *B*). Where the plasma membrane was cut perpendicularly, the binding of BPAG2-specific BP serum was more clearly observed along the plasma membrane. Gold particles were distributed both intracellularly and extracellularly across the plasma membrane of the hemidesmosome complex (Fig. 4 *C*). Affinity-purified anti-BPAG1 IgG bound only to the intracellular domain of hemidesmosome where the tonofilaments were inserted (Fig. 5 *A*), whereas affinity-purified anti–BPAG2 IgG bound exclusively along the plasma membrane of hemidesmosome (Fig. 5 *B*). In addition, BP serum reacting with both BPAG1 and BPAG2 labeled both the plasma membrane and the intracellular domain of the hemidesmosome complex, corresponding to the combined staining pattern of anti–BPAG1 and anti–BPAG2 (Fig. 5 *C*). The control antibodies showed no specific labeling (Fig. 5 *D*). When normal swine skin was used as the substrates, similar results were obtained with less background staining (Fig. 6).

Quantitative analysis of antigen distribution. The distances from the plasma membrane to the gold particles labeled by affinity-purified anti–BPAG1 IgG and affinity-purified anti–BPAG2 IgG were measured (Fig. 7). The distribution of the epitopes recognized by anti–BPAG1 IgG showed a single peak at 75 nm, and 80% of the labeling was distributed within 40–140 nm away from the membrane. The mean distance from the membrane was estimated as 91±5 nm (Fig. 7 *A*). The distribution of the epitopes recognized by anti–BPAG2 IgG showed a single peak at 0 nm and 80% of the labeling was distributed from within 10 nm extracellular to 50 nm intracellular. The mean distance from the membrane was estimated as 12±3 nm (Fig. 7 *B*). The distribution of the epitopes recognized by BP serum reacting with both BPAG1 and BPAG2 showed two peaks at 0 and 75 nm intracellularly (Fig. 7 *C*). The distribution of immunological epitopes of both BPAG1 (Fig. 7 *A*) and BPAG2 (Fig. 7 *B*) did not show normal distribution by the χ^2 test. There was a significant difference in distribution of immunological epitopes between BPAG1 and BPAG2 at a 0.01% significance level in Wilcoxon's rank sum test.

Discussion

In the present study, our novel immunoelectron microscopy technique, in conjunction with affinity-purification of autoantibodies, showed that there is a clear difference of reactivity in

the BMZ between the autoantibodies specific for BPAG1 and BPAG2. Namely, the epitopes of autoantibodies against BPAG1 located only in the intracellular domain of the hemidesmosome. In contrast, the epitopes of autoantibodies against BPAG2 located along the plasma membrane of the hemidesmosome complex both intracellularly and extracellularly. In that the thickness of the plasma membrane is 8–10 nm, the distribution of the epitopes of BPAG2 indicates that BPAG2 is a transmembrane protein. Only three patient sera were used in the present study. However, the result that affinity-purified anti-BPAG1 IgG and anti-BPAG2 IgG produced similar labeling patterns to BPAG1-specific BP serum and BPAG2-specific BP serum, respectively, suggests that the distribution of the epitopes of BPAG1 and BPAG2 shown in this study may have universal validity.

Immunoblotting for BP sera may occasionally show several minor proteins in addition to the two major BP antigens (12). However, it has been shown that there is a cross-reactivity either within a group of 240–200-kD proteins or within a group of 160–100 kD (33). Therefore, the 230-kD protein (BPAG1) and the 180-kD protein (BPAG2) are thought to be the major antigens for BP autoantibodies. Recently, the cDNA sequence data for both BPAG1 (15–18) and BPAG2 (19–21) have been reported. This allows the localization of BPAG1 and BPAG2 to be studied using monoclonal or polyclonal antibodies against fusion proteins obtained from the cDNAs (32). However, to clarify the ultrastructural localization of the immunogenic epitopes to which patient autoantibodies actually bind, it is necessary to use antibodies derived from BP serum. The total cDNA cloning of human BPAG1 has indicated the presence of a membrane-associated sequence of 17 amino acids near the carboxy terminus of BPAG1 (18). As the authors mentioned, if this sequence is indeed functional, ~ 80% of the molecule should be extracellular. However, an immunoelectron microscopic study using rabbit polyclonal antibodies against fusion protein indicated that the carboxy terminus of BPAG1 localizes in the intracellular domain of the hemidesmosome (32). The present study also indicated that BPAG1, or at least epitopes for autoantibodies, is present on the intracellular region.

There is some homology between the cDNAs for BPAG1 and desmoplakin I, a desmosomal plaque protein which is considered to be associated with intermediate filaments (17, 40). Desmoplakin I was shown to locate at the region where tonofilaments loop through the desmosomal plaque (41, 42). The similarity in topology between BPAG1 in the hemidesmosome and desmoplakin I in desmosome suggests that there may be some functional similarity between these molecules, i.e., BPAG1 may play a role in the attachment of the hemidesmosome to tonofilaments.

Recent studies of cDNA of human BPAG2 have shown that BPAG2 has 15 collagenous domains (21) with at least two membrane-associated segments (43), suggesting that it is a transmembrane protein (20, 21, 43). Our results of BPAG2 localization agree with this supposition. Another study dealing with the cloning of mouse BPAG2 cDNA showed a single Arg-Gly-Asp (RGD) sequence, suggesting the participation of this protein in cellular adhesion along with integrin (44). Integrin $\alpha 6/\beta 4$ was shown to locate in hemidesmosome by immunogold electron microscopy (45, 46). The localization of BPAG2 shown in this study was similar to that of integrin $\alpha 6/\beta 4$, and may indicate a relationship between these two proteins in cellular adhesion.

In BP sera, autoantibodies against BPAG2 are detected less commonly than those against BPAG1. However, only BPAG2 but not BPAG1, has autoantibody binding epitopes localizing extracellularly. Therefore, BPAG2 could be a target of BP autoantibodies in an uninvolved skin of BP patients. In other words, anti-BPAG2 antibodies may initiate primary change, and anti-BPAG1 antibodies may accelerate the BP lesions after plasma membrane suffers from some damages. We therefore feel that BPAG2 may play more important pathogenic role in BP than BPAG1 and that further extensive investigation should be focused on this molecule, which has to date been considered as a minor BP antigen.

In conclusion, we showed that there is a clear difference in the ultrastructural localization between the epitopes of BPAG1 and BPAG2, suggesting that these two molecules play different roles not only in dermoepidermal adhesion but also in the pathogenesis of blister formation in BP.

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