Two Groups of Bullous Pemphigoid Antigens Are Identified by Affinity-Purified Antibodies

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The bullous pemphigoid antigen was originally described as a 240-kD protein extracted from human epidermis, but a subsequent report has described patients' sera which react with epidermal proteins of molecular masses 240, 200, 180, 97, and 77 kD. We have evaluated the heterogeneity of the pemphigoid antigens identified by the sera of 10 patients with clinically typical bullous pemphigoid. We used indirect immunofluorescence and Western immunoblots of epidermal extracts prepared from epidermis separated by either 1 M salt or 20 mM EDTA to characterize the reactivity of both crude sera and affinity-purified antibodies. Affinity purification of antibodies was performed with either normal human epidermis or protein bands blotted onto nitrocellulose as immunoabsorbents.

The anti-basement membrane antibody titers determined by indirect immunofluorescence on the saline- and EDTA-separated epidermis were identical. Despite this, Western blots of extracts prepared from EDTA-separated epidermis demonstrated greater amounts of the 240-kD antigen than saline split skin. Multiple antigens were recognized in epidermal extracts on Western blots by most crude BP sera, including bands at 240, 200, 160, and 100 kD. Different sera reacted with these antigens with a markedly different intensity, falling into two major groups, those bearing antibodies to the 240–200-kD antigens and those with antibodies to the 160–100-kD components. When epidermis was used as a substrate for affinity purification of bullous pemphigoid antibodies, the eluted antibodies reacted with multiple bands on Western blots, demonstrating the reactivity of anti-basement membrane zone antibodies with multiple proteins. Antibodies eluted from several individual bands of immunoblots were found to react with the basement membrane on indirect immunofluorescence. When these nitrocellulose-purified antibodies were reapplied to Western blots, they cross-reacted within two groups, the 240–200 kD antigens and the 160–100 kD antigens. We conclude that pemphigoid antigens are best demonstrated when EDTA-split skin is used for extraction and that different pemphigoid sera may contain antibodies to two separate groups of basement membrane zone antigens. *J Invest Dermatol 94: 611–616, 1990*

Proteins extracted from skin which are antigenic in bullous skin diseases are now recognized in bullous pemphigoid (BP) [1,2], epidermolysis bullosa acquisita [3], pemphigus vulgaris [4], fogo salvagem [5], pemphigus foliaceus [6], and herpes gestationis [7]. In BP, initial reports of a putative antigen described a small peptide isolated from human epidermis [8] and from the urine of a BP patient [9] which could block antibody binding, as detected by indirect immunofluorescence. Subsequently two larger antigens, a 240–220-kD protein doublet, were immunoprecipitated by BP sera from lysates of keratinocyte cultures [10] and were extracted from suction blister-derived epidermis and identified on Western blot [1]. However, a more recent report has demonstrated that bullous pemphigoid sera react with multiple components of a crude epidermal extract, with molecular masses of 240, 200, 180, 97, and 77 kD [2]. It is not clear which of these proteins detected on electrophoretic immunoblots are responsible for the characteristic antibody binding to the basement membrane zone (BMZ) detected by indirect immunofluorescence or are responsible for the actual disease pathogenesis. One possibility is that these multiple components are breakdown products of a single epidermal antigen which might be responsible for the disease, although this is unlikely, given the pattern of reactivity of the sera [2]. Alternatively, there may be distinct antigens recognized by different sera, or some of the antigens may be ancillary to the disease process.

The question of antigen identity is of particular importance in BP as there is strong evidence that some circulating antibodies are pathogenic. It has been demonstrated that injection of IgG from patients with BP is pathogenic in vivo in a rabbit model [11] and that bullous fluid will cause blister formation in guinea pigs [12], thereby making it likely that BP is a disease caused by the humoral system.

In the experiments reported here, we have tested the hypothesis that the multiple antigens detected by immunoblot are the antigens responsible for positive indirect immunofluorescence. We used skin as an immunoaffinity substrate to elute anti-basement membrane zone (BMZ) antibodies. We also affinity purified antibodies to the

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Abbreviations:
ATPBS: albumin-Tween phosphate-buffered saline
BMZ: basement membrane zone
BP: bullous pemphigoid
EDTA: ethylenediaminetetraacetic acid
PBS: phosphate-buffered saline
PMSF: phenylmethyl-sulfonyl fluoride
SDS: sodium dodecyl sulfate
ZIA: basement membrane zone immunoaffinity

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different molecular weight components using Western blots. We have used these affinity-purified antibodies to address the relationship of the antigens detected on Western immunoblots and the antigens detected by indirect immunofluorescence. We also looked for cross-reactive antibodies to the multiple antigenic proteins detected on immunoblots.

MATERIALS AND METHODS

Sera Sera were studied from 10 consecutive patients with clinical features of BP who had excess sera available from our immunodiagnostic laboratory. All sera had titers of bullous pemphigoid autoantibodies detected by indirect immunofluorescence with monkey esophagus substrate ranging from 1:80 to 1:10,240. Random sera provided by the blood bank were used as controls.

Epidermal Extracts Epidermis was separated from dermis at the basement membrane zone by two methods. Abdominoplasty skin, keratomed to a depth of 0.5 mm, was stored frozen and then either: 1) incubated in 20 mM ethylenediaminetetraacetic acid (EDTA), 10 μM phenylmethylsulfonyl fluoride (PMSF) in phosphate-buffered saline (0.126 M NaCl, 0.008 M Na_2HPO_4, and 0.002 M KH_2PO_4, pH 7.2) (PBS); or 2) incubated in 1 M NaCl, 1 mM EDTA, and 10 μM PMSF. Both incubations were carried out at 4°C for 16–20 h. The epidermis was then easily separated from the dermis and homogenized in 1% sodium dodecyl sulfate (SDS) containing 5% beta-mercaptoethanol following the procedure of Stanley et al [1]. After being heated to 95°C for 3 min, the mixture was cooled and centrifuged, and the supernatant was frozen at −70°C and used within 5 months. There was no loss or change in antigenicity with storage.

Electrophoresis and Blots Eighty micrograms of epidermal extract protein was used for each lane of polyacrylamide gel electrophoresis for both EDTA and saline extracts. Protein determinations were performed by slight modifications of the method of Lowrey [13] and electrophoresis was performed by slight modifications of the method of Laemmli, with use of an 8% acrylamide running gel, a 5% acrylamide-stacking gel, and a 1:30 acrylamide to N,N′-methylene-bisacrylamide ratio [14]. Samples were heated for 5 min at 95°C in the presence of fresh beta-mercaptoethanol before being loaded on the gels. Following electrophoresis, proteins were transferred electrophoretically onto nitrocellulose, as described by Towbin et al [15]. The incubation to block nonspecific protein binding to the nitrocellulose used 0.5% bovine serum albumin and 0.05% Tween-20 in PBS (ATPBS). Individual lanes were incubated for 2 h at room temperature with sera diluted 1:50 in ATPBS, affinity-purified anti-BMZ antibodies for 2 h at room temperature, or with Western blot affinity-purified antibody for 3 h. After three washes in ATPBS, each lane was incubated for 30 min with biotinylated goat anti-human IgG diluted 1:200 in ATPBS (Vector). This was followed by three washes, a 30-min incubation with an avidin-biotin-peroxidase complex, and staining with 4-chloronaphthol and H_2O_2 [16].

Basement Membrane Zone Immunoaffinity (ZIA) EDTA-split epidermis was used as an immunoaffinity substrate for isolation of anti-basement membrane zone antibodies. Sera from patients and controls were diluted 1:20 in PBS and applied to the epidermis for 2 h. Following three 10-min washes in PBS, the antibodies were eluted with 20 mM sodium citrate buffer, pH 3.2, at 37°C. The eluate was exchanged at 4, 8, 12, 16, and 24 h. Each wash was immediately individually neutralized with appropriate quantities of 2 M Tris buffer, pH 8.0. The five eluates were pooled, dialyzed against distilled water, and lyophilized. The product was reconstituted in PBS for immunofluorescence, and with Tween-20 and PBS for Western blots.

Western Blot Immunoaffinity Protein bands on nitrocellulose blots of Laemmli gels were also used as immunoaffinity substrates for purification of antibodies directed against specific proteins in the epidermal extract, as described by Olmsted [17] and modified by Smith and Fisher [18]. Vertical lanes from both sides of the nitrocellulose imprint of the gel were cut and immunochemically stained with sera for identification of the bands of interest. Horizontal strips of nitrocellulose containing the antigen of interest, but not exposed to sera, were then cut, and sera were applied as 1:25 or 1:50 dilutions in ATPBS for 2 h. After a wash in ATPBS, the antibodies from horizontal strips from blots of two gels were eluted in 600 μl 20 mM sodium citrate, pH 3.2, 0.05% Tween-20 for 30 min at 37°C and were immediately neutralized with Tris, as described above. These affinity-purified antibodies were used for immunofluorescence and Western blots without further concentration.

Immunofluorescence Indirect immunofluorescence was performed on three substrates: monkey esophagus (IF Testing Service, Buffalo, NY), EDTA-separated epidermis, and 1 M NaCl-separated epidermis. Isolated epidermis was embedded in OCT (Miles Scientific) and 6-μ sections were cut at −23°C. Slides were allowed to air dry at room temperature for 20 min and then were rinsed with PBS. The slides were then exposed to serial dilutions of sera in PBS for 30 min or affinity-purified antibodies for 2 h at room temperature in a humid environment. Following a 30-min wash in PBS, fluorescein-conjugated goat anti-human IgG (IgG fraction, 31.7 mg/ml protein, 4.09 mg/gm fluorescein to protein ratio, Cappel) diluted 1:80 in PBS was applied for 30 min at room temperature. The slides were again washed in PBS and evaluated on a Leitz epifluorescence microscope. Titers of sera were recorded as the last dilution with visible antibody binding.

Several specific antibodies were used for studies to characterize the location of separation of EDTA- and saline-split skin. Mouse monoclonal antibody to laminin (Calbiochem) was used at a dilution of 1:50 and the second antibody was fluorescein-conjugated goat anti-mouse IgG (IgG fraction, 13.5 mg/ml protein, 4.72 mg/gm fluorescein to protein ratio, Cappel) diluted 1:80 in PBS. Mouse monoclonal antibody to type IV collagen (Chemicon) was used at a dilution of 1:200 in PBS and the second antibody was fluorescein-conjugated goat anti-mouse IgG diluted 1:80 in PBS. Epidermolysis bullosa acquisita serum, a kind gift from David Woodley, M.D., was diluted 1:200 in PBS. This serum has been previously shown to react with the 290-kD type VII collagen [3]. The second antibody was goat anti-human IgG, as described above. Mouse monoclonal antibody to fibronectin (Calbiochem) was diluted 1:80 and used with fluoresceinated goat anti-mouse, as above. All primary and secondary incubations were at room temperature for 30 min.

RESULTS

Pemphigoid Antigens in Epidermal Extracts Antigens detected in epidermal extracts on Western blots by the 10 patient sera were heterogeneous. Many reacted with the previously described 240-kD band, while others reacted with the lower-molecular-weight bands and some with both. However, the staining intensity of different bands changed dramatically when the method of separation of epidermis from dermis was changed from incubation in 1 M salt to incubation in 20 mM EDTA, as seen in Fig 1. The large number of background bands is due to our use of low (1:50) dilutions of sera. In the region below 66 kD, sera from BP patients and normals all had staining, presumably due to keratin auto-antibodies and the heavy chain of the IgG extracted from the epidermis. In the extract prepared from epidermis which had been split from the dermis after a sodium chloride incubation, the 240-kD antigen was barely detectable at 1:50 dilutions of any of the patients' sera. However, in the extract of epidermis which had been separated with 20 mM EDTA, the 240-kD antigen was detected by nine of the 10 patient sera. In addition, other discrete antigens, including major bands at 200, 160, and 100 kD, could be easily identified. The major band at 160 kD was identified by five of the 10 sera, and these sera were generally the same ones which had minimal reactions with the 240-kD component, as is shown in Fig 1*B*.

Because the method of epidermal separation produced a marked difference in serologic reactivity to immunoblotted epidermal antigens, we sought to determine if the immunofluorescent titers of the

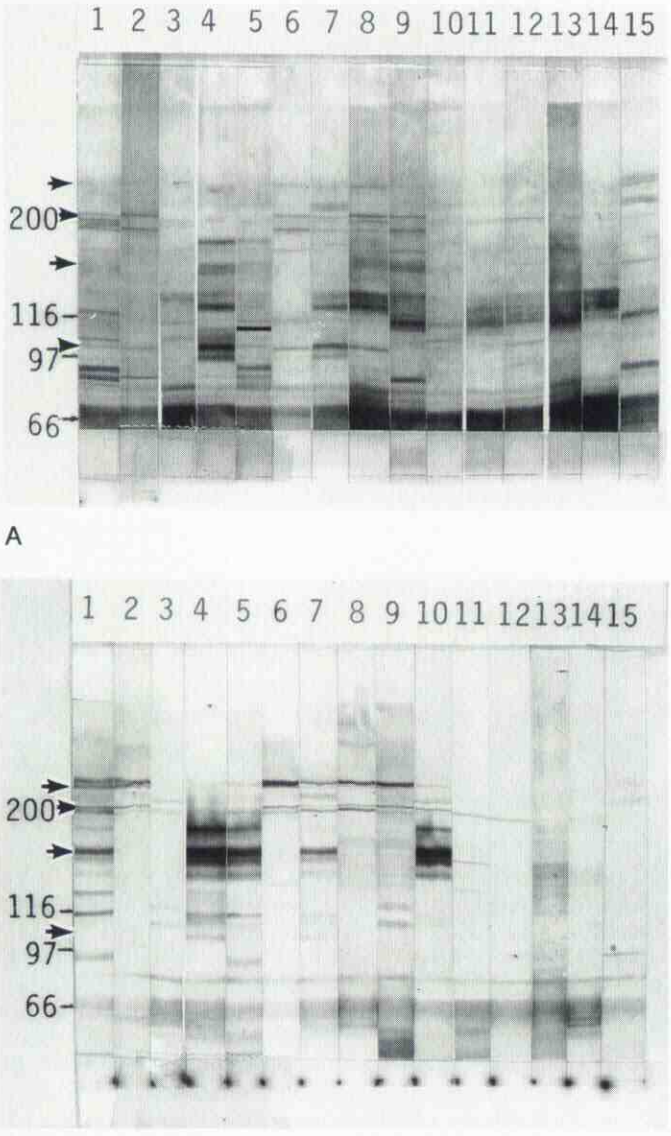


Figure 1. Western blots of 1 M NaCl (*A*) and 20 mM EDTA (*B*) separated extracted epidermis with BP and normal sera. For each similarly numbered *lane* in the two panels, the sera are the same. *Lanes 1–10* are BP sera, *lanes 11–15* are normal sera. Migration of molecular-weight markers is shown at the left and the *arrows* represent the approximate positions of the 240-, 200-, 160-, and 100-kD antigens. The antigens are poorly seen in *A* but the 240- and 160-kD antigens are clearly seen in *B*.

sera against the BMZ also varied with the method of epidermal separation. Table I shows the titers of the 10 BP sera against EDTA- and saline-split epidermis. The titers of each serum were within one $\log_2$ dilution when the two substrates were compared. Whether the serum identified predominantly the 240- or the 160-kD antigen on Western blot, the staining pattern was the same, with equally avid binding to the BMZ of the sectioned epidermis (data not shown). Further differences between the methods of separation were sought by immunofluorescence mapping with a variety of antisera. There were no significant differences between the two methods in location of antibody binding by antisera to type IV collagen, fibronectin, elastin, laminin, and type VII collagen (epidermolysis bullosa acquisita serum).

Immunoaffinity-Purified Sera To characterize which, if any, of the antigens detected on immunoblot correspond with the antigen detected by immunofluorescence, we used EDTA-split epidermis as an immunoaffinity substrate for purification of antibodies. BP and control sera were applied to a large sheet of EDTA-separated epidermis, and the bound antibodies were eluted with citrate, as described in *Materials and Methods.* These ZIA-purified antibodies were used to probe electrophoretic blots for identification of eluted basement membrane zone antigens present in the epidermal extract. Affinity-purified antibodies were compared to the whole serum of the same patient, and their reactivity with epidermal extract components is shown in Fig 2. In Fig 2*A*, those sera which had a prominent reaction with the 240-kD antigen are shown alongside the ZIA-purified antibody. In Fig 2*B*, those sera which reacted primarily with components of the epidermal mixture other than the 240-kD antigen are seen. In all cases, there is an enhancement of the same bands identified by the whole sera, demonstrating that the antibody binding to the BMZ can bind to multiple components of the extract of the epidermis. As a control, ZIA-purified antibody was reapplied to EDTA-split epidermis and, by indirect immunofluorescence, bound along the BMZ (data not shown.)

As a second method of addressing the question of the relationship of antigens detected by immunofluorescence and Western blot, we used nitrocellulose blots as an affinity substrate for antibody purification and then tested the affinity-purified antibodies for their epidermal binding characteristics. The antibodies eluted from the bands at 240, 200, 160, and 100 kD of epidermal extract reacted with the basement membrane zone of EDTA-split epidermis by indirect immunofluorescence at 2+, 0, 3+, and 2+, respectively (Fig 3). Control elutions from strips of nitrocellulose incubated with patient sera were negative by indirect immunofluorescence. These results demonstrate that the 240-, 160-, and 100-kD molecular weight components are antigens present at the BMZ.

To address the possible interrelation of epidermal antigens of molecular masses of 240, 200, 160, and 100 kD, we reapplied the nitrocellulose affinity-purified antibodies to a nitrocellulose blot of the epidermal extract (Fig 4). The antibodies eluted independently from the 240- and 200-kD bands cross-reacted while showing a preference for the band from which they were eluted. The antibodies eluted from the 160- and 100-kD bands also show strong cross-reactivity with each other and a slight reaction with the 200-kD band, but no reaction with the 240-kD band. The affinity purification of antibodies on nitrocellulose has been repeated a minimum of two times for each band with different patient sera, with similar results. From these studies, we conclude that there are at least two groups of antigens associated with BP; antibodies affinity purified from patient sera to the 240-kD and 160-kD antigens do not cross react, and both react with the BMZ.

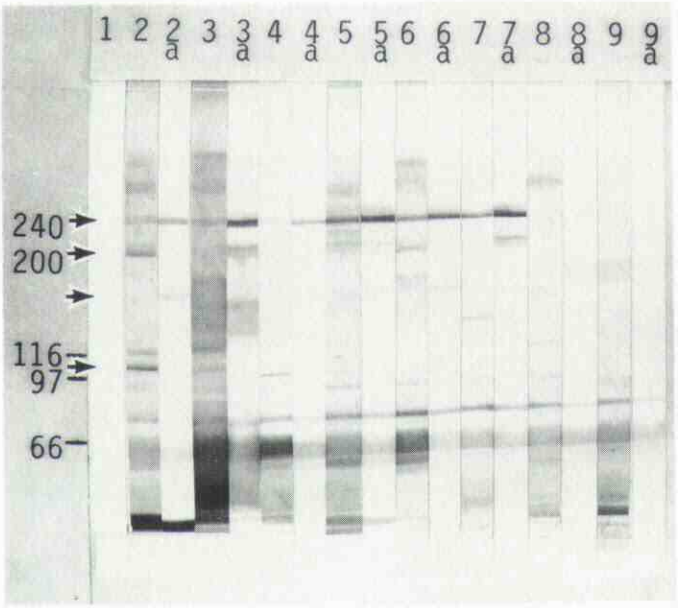
Table I. Indirect Immunofluorescence Titers Using Saline- and EDTA-Separated Skin as Substrates[a]

Patient Number	Saline	EDTA
1	640	640
2	80	160
3	320	640
4	640	1,280
5	10,240	5,120
6	1,280	1,280
7	1,280	2,560
8	1,280	1,280
9	2,560	1,280
10	320	160

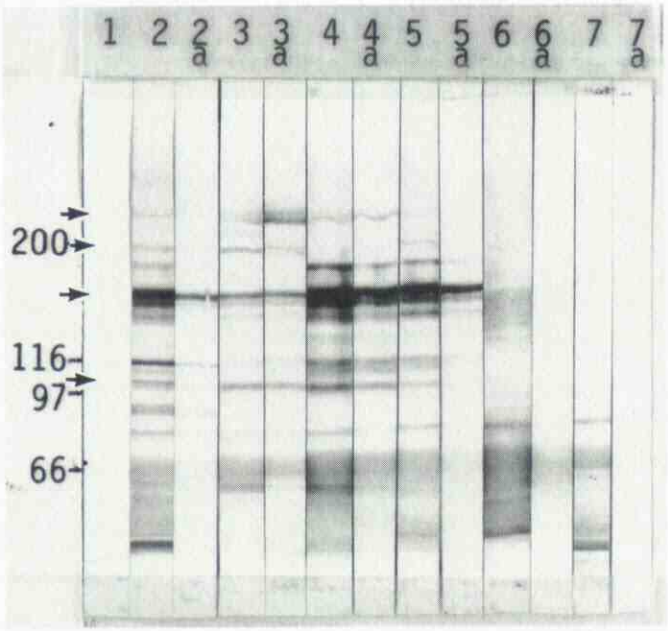
[a] Patient numbers correspond to the lane numbers in Fig 1. The number shown is the reciprocal of the dilution of the sera.

DISCUSSION

We have demonstrated that BP sera recognize antigens of multiple molecular weights in crude epidermal extracts of both 20 mM EDTA- and 1 M NaCl-split skin. However, the expression of these antigens varies with the method of epidermal separation. Moreover, we have confirmed the importance of these antigens by demonstrat-



A



B

Figure 2. Western blots of whole sera and ZIA-purified sera against EDTA-separated epidermal extract. *Lane 1* shows no primary antisera in both panels. In each case the ZIA serum, labeled with an "a," is matched with the unfractionated serum with the same number in the preceding lane. In *A*, those sera reacting with the 240-kD component are shown. *Lanes 2–7* are BP sera, *lanes 8* and *9* are normal sera. In *B*, those sera reacting primarily with the 160-kD component are shown. *Lanes 2–5* are BP sera, *lanes 6* and 7 are normal sera.

ing that they are components of the basement membrane using affinity-purified antibodies. The antigens fall into two general groups. The same groups were observed by general description of the Western blot analysis of patient sera and by Western blot analysis of patterns of cross-reactivity of affinity-purified antibodies.

The observation that different patient sera demonstrated different patterns of reactivity is similar to that of Labib et al [2]. This result cannot be explained by variabilities in extraction, preparation, or degradation, because all sera were exposed to the same preparation of epidermal antigens extracted, electrophoresed, blotted, and developed simultaneously. The 240-kD antigen initially described by Stanley et al [1] is commonly seen. Another frequently observed antigen was the 160-kD component. The component at 160 may be identical to the 180-kD antigen described by Labib et al and the 166-kD antigen of Mueller et al [19], with the differences in apparent molecular weight due to the differing cross-link concentrations used in the running gels; Labib et al [2] used a 1 : 60 acrylamide to bis-acrylamide ratio while our ratio was Laemmli's original 1 : 30 [14]. Changes in this ratio can cause migration differences [20]. Alternatively, the 160-kD antigen could be a proteolytic fragment of the 180-kD antigen.

There is an apparent relation between the multiple antigens detected on Western blot. Antibodies affinity purified from each molecular weight band cross-reacted in two major groups. These groups were the 240–200-kD bands and the bands in the 160–100 region, the same groups into which the sera can be divided by pattern of reactivity. Overall, we interpret this as evidence for two antigen groups. However, proteins in a denatured form on Western blots and in vivo may express different antiseric determinants as is demonstrated by the cross-reactivity of affinity-purified antibodies to the 200-kD antigen and the 240-kD antigen on blots, but the lack of reactivity of affinity-purified antibodies to the 200-kD antisera with the basement membrane. Similarly, patient sera with low titers to the large BP antigen on Western blots have been shown to immunoprecipitate intact antigen from keratinocyte lysates [19]. Therefore, lack of reactivity on blots cannot eliminate the possibility that a serum contains antibodies to a nondenatured antigen, or that an antigen is not exposed in an immunofluorescence assay.

The relationship of the multiple antigens observed on Western blots could have several other theoretical explanations, including relationship by general degradative proteolysis, by specific cleavage during assembly, or by post-translational modification such as glycosylation. If specific or nonspecific proteolysis were the sole factor generating multiple bands, we would expect that most antisera would recognize a similar pattern of antigens on any extract and that antibody directed at a lower-molecular-weight component would generally react with a higher-molecular-weight component. This was the pattern observed within the 160- and 100-kD antigen group, making a proteolytic relationship likely, but it was not the case when the 240–200-kD group was compared with the 160–100-kD group. Our extraction conditions include both PMSF and EDTA similar to the conditions used by Stanley and coworkers [1,19], but not pepstatin or antipain, leupeptin, and chymostatin, as employed in the procedure of Labib et al [2]. If proteolysis were the way in which the 160-kD antigen was generated from the 240-kD

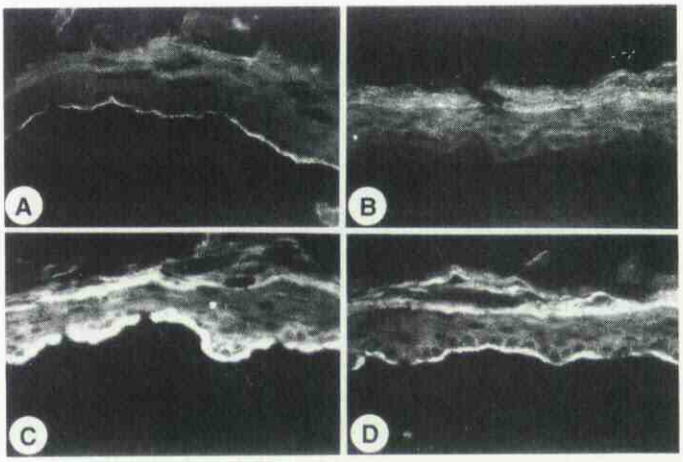


Figure 3. Indirect immunofluorescence with sera affinity purified from Western blots. All sera were reacted with EDTA-split epidermis. The panels differ in which molecular weight band was used to purify the antibody. *A*: 240 kD; *B*: 200 kD; *C*: 160 kD; *D*: 100 kD. *A*, *C*, and *D* show positive BMZ fluorescence but *B* is negative.

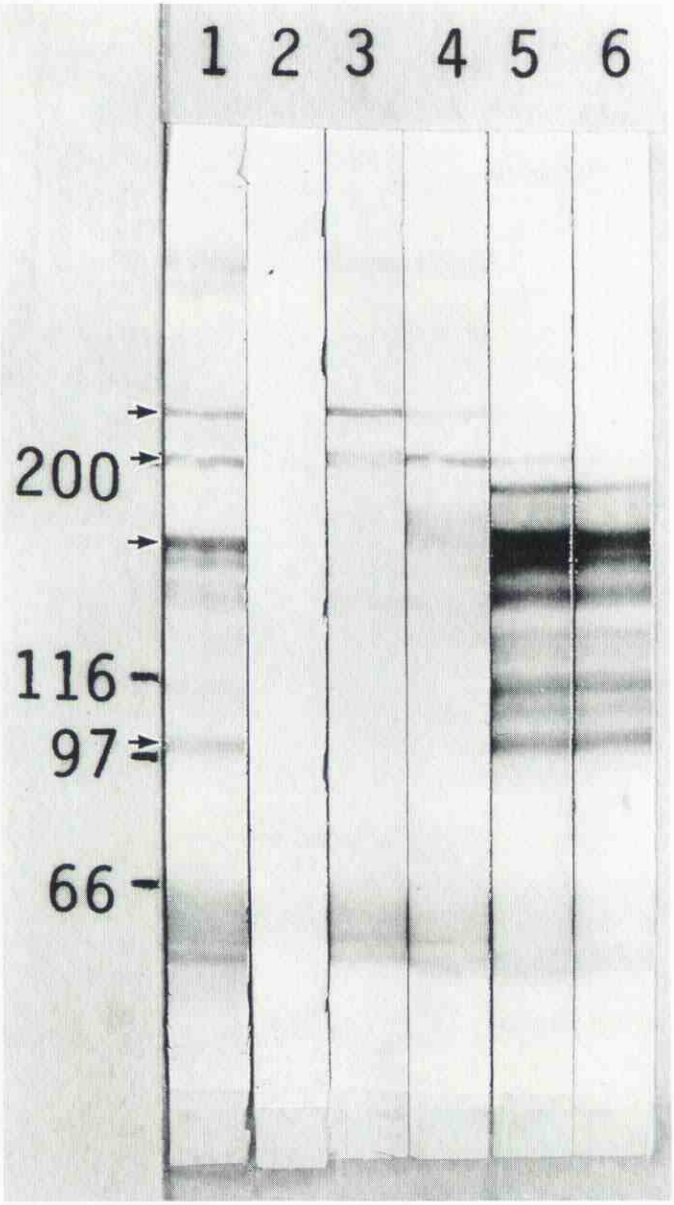


Figure 4. Western blots with antibodies purified from Western blots. All are blotted against EDTA-separated epidermal extract. The lanes differ in which molecular weight band was used to purify the antibody. *Lane 1:* whole serum for reference, showing the 240-, 200-, 160-, and 100-kD antigens. *Lane 2:* control extraction of non-blotted, blocked nitrocellulose. *Lane 3:* 240 kD. *Lane 4:* 200 kD. *Lane 5:* 160 kD. *Lane 6:* 100 kD.

antigen, it would require that virtually all common antigenic determinants in the larger be hidden, such as by protein folding or sugars, for one to explain those sera and affinity-purified antibodies which react with the 160-kD but not the 240-kD antigen. We consider both protein folding on SDS gels under denaturing conditions and glycosylation unlikely, because there is evidence that these antigens are not glycosylated [20,21]. Furthermore, because some patient sera do not react in a detectable fashion with the 240–200-kD antigens, the antigenic determinants on the 160-kD antigen must be exposed in vivo for explanation of the disease in these groups.

It is our experience that on any Western blot of a crude antigen mixture, at low dilutions, some reaction with many sera will occur. One way of avoiding this is to dilute the sera until only one band is visible. This approach carries the unstated assumptions that the most abundant antibody is most important and that more dilute antibodies or less abundant antigens in the extract are not significant. It seems plausible that low-titer antibodies directed at a rare or poorly extractable antigen could still result in a great amount of in vivo immunoglobulin deposition with time and result in disease. It is for this reason that we used relatively low dilutions of patient sera to develop our blots (1:50 dilutions), compared to those used by others who have studied BP, such as of 1:100–1:200 [2] or 1:250 [1]. The higher dilutions of Stanley et al [1] may in part explain the absence of the lower-molecular-weight antigen recognition by his sera.

The antigens isolated by the two methods, EDTA and saline separation of the epidermis, are markedly different. We initially noted this in our laboratory as a serendipitous finding, having used both methods of separation for different purposes. A possible explanation is that there is a greater amount of the 240-kD pemphigoid antigen preserved in the EDTA-split epidermis; however, there were no differences in indirect immunofluorescence titers between the two substrates, nor was the saline-split dermis stained with patient sera, as would be expected if some of the 240-kD antigen remained with the dermis upon saline separation. This difference could be due to proteolysis, differential extraction efficiency, or separation at a different point in the basement membrane. The localization of BMZ separation by the two methods was very similar, as assessed by immunofluorescence mapping. Previous studies have used suction blister cleavage [1,22], saline splitting [22,23], heat separation [2,22], KSCN separation [8], thermolysin treatment [24], and EDTA separation [25] for study of epidermis. If results of different laboratories are to be compared, these methods obviously need to be similar. Moreover, the optimal method for isolation of any given component needs to be established, and there may not be a single best method. This is highlighted by the observation that saline- and EDTA-separated epidermis are equivalent as substrates for indirect immunofluorescence, but there is a striking difference in the extracted antigens. We feel that for the evaluation of BP sera, the EDTA separation is superior to the saline method for extraction, but this may not be the case for other antigens.

An obvious question raised by these results is the relationship of clinical disease to the antigens recognized. Because the majority of these sera were mailed to our laboratory, it has thus far been impossible to obtain clinical follow-up on those BP patients. We are now studying more patients prospectively to assess if differences in presentation or responses to treatment correlate with the pattern of seroreactivity.

In summary, we have demonstrated that there are two classes of antigens associated with BP. Some patients seem to have antibodies to only one class as detected by immunoblotting, but several have some antibodies to both classes. The two antigens each have multiple molecular weight forms in crude extracts. The definition of the relationship between the two antigen groups and the members of each group will require further investigation, but both appear to be independently associated with BP and therefore candidates for antigens involved in the pathogenesis of the disease.

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ANNOUNCEMENT

The 20th Annual Meeting of the European Society for Dermatological Research will be held from June 9 to 12, 1990 at the Centro Congressi Torino Esposizion, Torino, Italy. For further information please contact: 1) Gunhild L. Vejlsgaard, M.D., Secretary, ESDR, Department of Dermatology, Bispebjerg Hospital, Bispebjerg Bakke 23, DK-2400 Copenhagen NV, Denmark; 2) Maria Grazia Bernengo, M.D., ESDR Board Member, Clinica Dermatologica dell'Universita di Torino, Via Charasco 23, I-10126 Torino, Italy.