0022-202X/83/8102-0149$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:149–153, 1983

Vol. 81, No. 2
Printed in U.S.A.

Localization of Basement Membrane Components After Dermal-Epidermal Junction Separation*

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Adult human skin was separated at the dermal-epidermal junction (DEJ) by 4 published methods that involved different mechanisms of action: cold 1 M salt (tissue extraction), cold trypsinization (enzymatic), induction of a suction blister (mechanical), and warm phosphate-buffered saline (protease activation). The localization of DEJ macromolecules was studied after each separation method. By all of the methods tested, bullous pemphigoid antigen remained closely associated with the epidermis while laminin, the basement membrane heparan sulfate proteoglycan, and collagen types IV and V remained with the dermal side of the separation. The bullous pemphigoid antigen is, then, the DEJ component most closely associated with the epidermal basal cell. Of the basement membrane components tested, only the basement membrane heparan sulfate proteoglycan was trypsin-sensitive.

The basement membrane within the dermal-epidermal junction (DEJ) is known to contain several macromolecules including the bullous pemphigoid antigen (BPA), a large glycoprotein called laminin, a heparan sulfate-rich proteoglycan, and type IV collagen [1]. Other components of some basement membranes may include fibronectin and type V collagen, but the exact localization of these two macromolecules is less certain [2–8]. It is unknown whether these macromolecules play a role in dermal-epidermal adhesion. The purpose of this study was to separate adult human skin at the DEJ using 4 different published methods and study the localization of BPA, laminin, the basement membrane proteoglycan, and collagen types IV and V by indirect immunofluorescence (IIF) with highly purified antibodies to these known DEJ macromolecules and serum from bullous pemphigoid (BP) patients. The 4 methods of DEJ separation used here are known to separate whole skin through the lamina lucida area of the basement membrane zone (BMZ), leaving the lamina densa with the dermis. Further, the mechanisms of separation are thought to be different: mechanical (suction blister formation), enzymatic (cold trypsinization), connective tissue extraction (cold 1 M NaCl), and activation of proteases (warm phosphate-buffered saline, PBS). We found that the BPA tends to adhere to the epidermis after DEJ separation while the other major macromolecules tend to remain with the dermis.

Manuscript received June 15, 1982; accepted for publication February 2, 1983.

* This paper was presented in part at the National Meeting of The Society for Investigative Dermatology, Washington, D. C., May 6–8, 1982.

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Abbreviations:
BMZ: basement membrane zone
BP: Bullous pemphigoid
BPA: bullous pemphigoid antigen
DEJ: dermal-epidermal junction
IIF: indirect immunofluorescence
MEM: minimal essential medium
PBS: phosphate-buffered saline

MATERIALS AND METHODS

Skin Samples

Suction blisters were induced in situ in 3 normal adult volunteers (see below). For the other 3 separation methods, adult human skin from fresh surgical specimens were used. The skin was first keratotomed with a Castroviejo keratome set at 0.4 mm and washed once in Eagle's minimal essential medium (MEM). After each separation technique mentioned below, the epidermal and dermal components of the skin were washed in cold PBS, embedded in O.C.T. (Lab-Tek Products), quick-frozen in liquid nitrogen, and kept at −20°C. Within a week, they were cut into 6-μm sections on a cryostat microtome and subjected to the immunofluorescent procedure.

Skin Separation Techniques

The DEJ separation methods used were: (1) *Cold trypsinization*: the technique of Medawar [9] was used. Keratotomed skin was floated on precooled 0.25% trypsin (GIBCO no. 610.5050) and kept at 4°C for 18 h. The epidermis was then easily separated from the dermis in MEM with 10% fetal calf serum. (2) *Cold salt*: the method of Scaletta and colleagues [10] was followed. Keratotomed slices of skin were rocked in 1 M NaCl at 4°C for 72–96 h. At this time, the epidermis was easily removed from the dermis. (3) *Warm saline*: the DEJ separation as described by Regnier and colleagues [11,12] was employed. Keratotomed skin slices were floated in PBS at 37°C for 72–96 h, at which time the epidermis was readily peeled off of the dermis. (4) *Induction of a suction blister*: suction blisters were induced in 3 normal adult volunteers using a suction blistering device as described by Kiistala [13]. After the skin was blistered, a 4-mm punch biopsy enveloping the blister was performed and the specimens were then quick-frozen and processed for IIF.

Sera

Three sera from BP patients were used. By IIF, these sera stained the BMZ of normal human skin and monkey esophagus and had a titer of at least 1:80. A working dilution of 1:10 was used. In addition to activity against the BMZ, 1 BP sera also had antibody against the cytoplasm of maturing keratinocytes (U-Cyt) as described by Bystryn et al [14] and Saurat et al [15] with a titer of 1:80.

Affinity column-purified antibodies to laminin, the basement proteoglycan, and collagen type IV have been previously well characterized [16–18] and were used in this study at a working dilution of 1:50.

Type V collagen antibodies were raised in rabbits using purified bovine type V collagen prepared from placental membranes as described by Glanville and Kuhn [19]. The migration pattern of the purified collagen on sodium dodecyl sulfate-polyacrylamide gels was characteristic of type V collagen and distinct from other collagen types. The antibodies to type V collagen were affinity column-purified and were shown to cross-react with human type V collagen but not with other known basement membrane components [20].

By direct immunofluorescence, these purified antibodies stain the basement membranes around vessels and between the epithelium and connective tissue of a variety of tissues including human skin and monkey esophagus (Fig 1).

Blocking studies were performed with the antiserum to type V collagen by incubating 150 μl of the antiserum overnight at 4°C with 10 μg of laminin, type IV, or type V collagen before the IIF procedure in the morning. Normal human, goat, and rabbit sera served as controls in each IIF experiment depending upon the species immunized in obtaining the experimental sera.

Indirect Immunofluorescence

Six-micron cryostat sections of epidermis and dermis were washed for 15 min in cold PBS twice, and subjected to the IIF chain as previously described [21] with appropriate fluorescein isothiocyanate conjugates (Cappel Laboratories—goat antirabbit IgG for antiserum to the heparan sulfate proteoglycan and type V collagen, rabbit antigoat IgG for laminin and type IV collagen antiserum, and goat antihuman IgG for BP serum). Semiquantitative studies were performed with BP serum by serially titering out the serum from 1:10 to 1:320 and staining sections of epidermis and dermis by IIF after the 4 separation methods.

Electron Microscopy

After DEJ separation, epidermis and dermis were examined by electron microscopy after fixation [22], dehydration, and processing in the routine manner.

RESULTS

Indirect immunofluorescent staining of skin or esophagus with purified antibody to type V collagen stained the basement membrane around blood vessels and between the epithelium and connective tissue (Fig 2*A*). Immunofluorescent staining was also evident around the fascicles of the esophageal smooth muscle bundles (Fig 2*B*). This staining could not be extinguished by preincubation of the antibody with laminin or type IV collagen, but was readily blocked with type V collagen.

The results of the DEJ separation experiments are summarized in Table I. The antigenicity of the basement membrane heparan sulfate proteoglycan was lost from both the epidermis and dermis after separation of the skin by cold trypsinization (Fig 3). Laminin, type IV collagen, and type V collagen, however, remained antigenic and were detected by their corresponding antibodies as bright linear staining along the BMZ of the dermis and around dermal blood vessels. As with trypsinization, when the DEJ was separated by cold salt, warm PBS, or the induction of a suction blister, these macromolecules remained with the dermis and demonstrated similar fluorescent staining patterns (Figs 4, 5). The antigenicity of the heparan sulfate proteoglycan was conserved by these DEJ separation techniques and remained with the dermis, staining the BMZ and around blood vessels similarly to laminin and collagen types IV and V. Except for laminin, to a limited degree (see below) these matrix molecules were not detected on the polar aspect of the basal keratinocytes by these separation techniques.

TABLE I. *Localization of dermal-epidermal junction components after separation of whole skin by various techniques*

	Bullous pemphigoid antigen	Laminin	Type IV collagen	Type V collagen	Heparan sulfate proteoglycan
Warm PBS	E (1/10)	D/e	D	D	D
Cold salt	E (1/320)	D/e	D	D	D
Trypsin	E (1/320)	D/e	D	D	N
Blister	E (1/320)	D/e	D	D	D

E = epidermal, D = dermal, D/e = predominantly dermal, N = none, and parentheses in the BPA column are the sera dilutions.

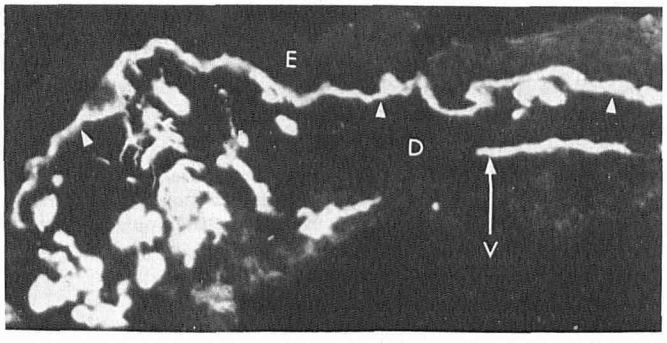

FIG 1. Indirect immunofluorescence of human adult skin with purified antibody to heparan sulfate-rich basement membrane proteoglycan. The antibody stains the basement membrane between the epidermis and dermis (*arrowheads*) and around dermal vessels (*arrow—V*). E = epidermis, D = dermis, V = vessel (× 250).

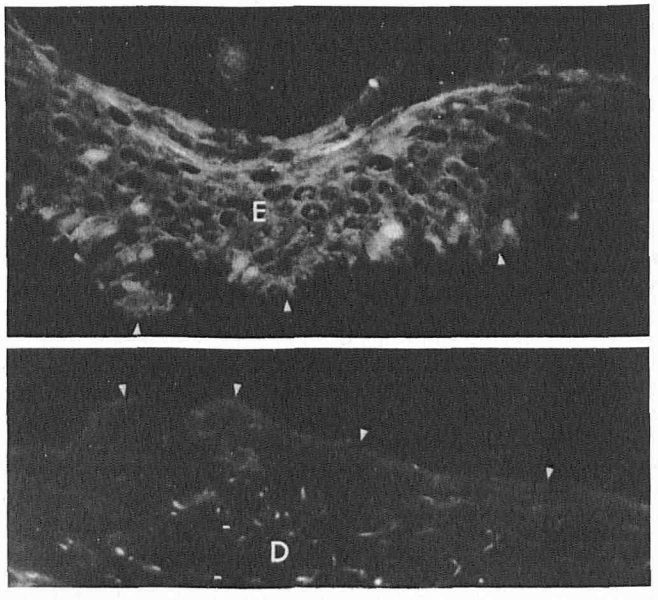

FIG 3. Indirect immunofluorescent staining with purified antibody to the heparan sulfate-rich basement membrane proteoglycan of human epidermis (*E*) and dermis (*D*) isolated by cold trypsinization. Compare with Fig 1, before trypsinization. *Arrowheads* point to the polar aspect of the epidermal basal cells and the BMZ of the dermis (× 250).

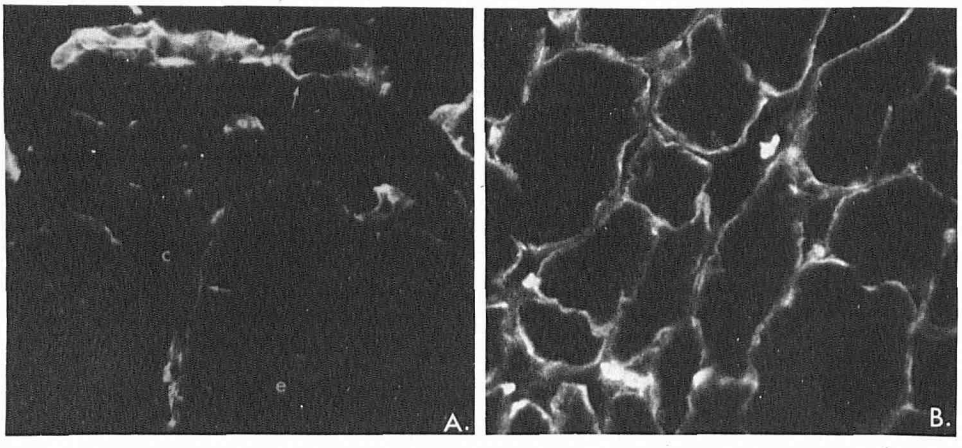

FIG 2. Indirect immunofluorescent staining of monkey esophagus sections stained with purified antibody against type V collagen that had been preincubated with 10 μg of pure type IV collagen (*A*). Similar results occurred when the type V antibody was preincubated with 10 μg of laminin. Type V collagen was also localized around the fascicles of the esophageal smooth muscle bundles (*B*). The *arrows* point to the BMZ around blood vessels and between the epithelium (*e*) and connective tissue (*c*) (× 250).

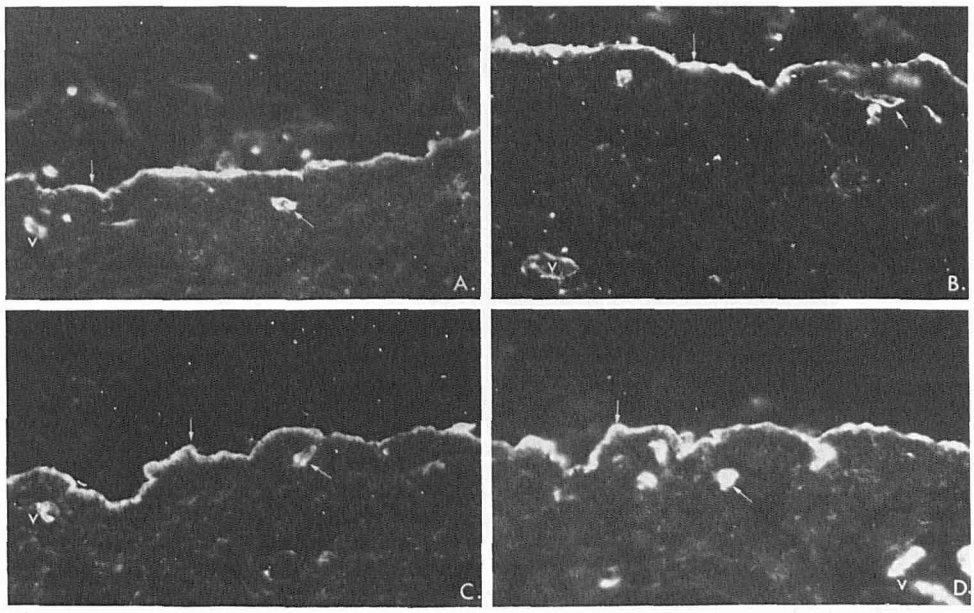

FIG 4. Indirect immunofluorescence of a suction blister base (dermis) stained with purified antibodies to laminin (*A*), type IV collagen (*B*), the heparan sulfate-rich basement membrane proteoglycan (*C*), and type V collagen (*D*). *Arrows* point to the BMZ of the blister base and to dermal blood vessels (*v*) (× 250).

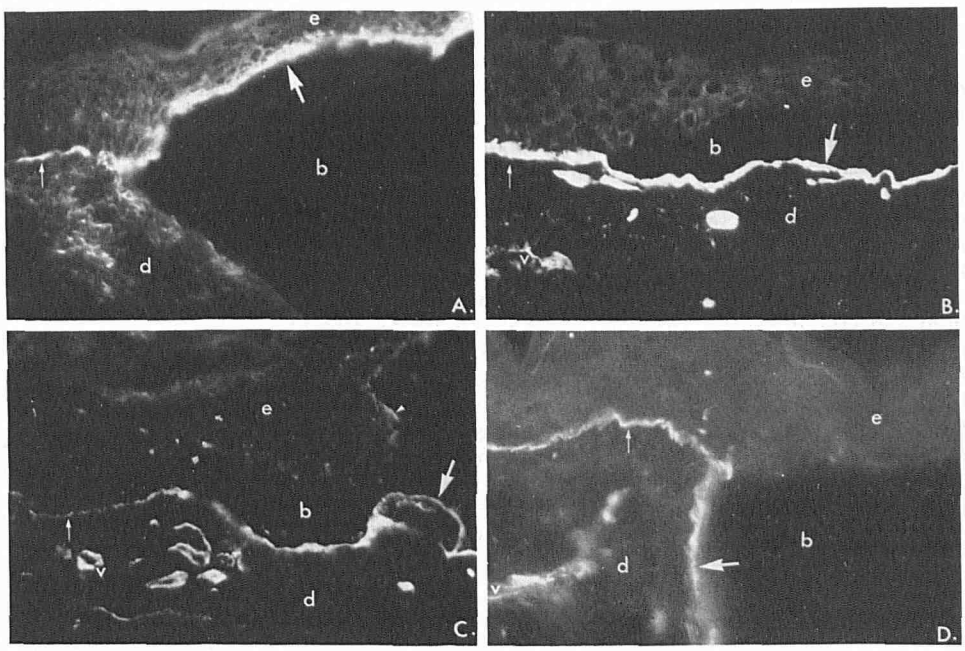

FIG 5. The junction of a suction blister stained by IIF with bullous pemphigoid antibody (*A*), purified antibody to type V collagen (*B*), laminin (*C*), and type IV collagen (*D*). *Small arrows* point to the intact skin. *Large arrows* point to the basement membrane component within the DEJ separation (*b* = blister space, *e* = epidermis, *d* = dermis, *v* = vessel). Note in *C* specks of fluorescence on the polar aspects of the epidermis (*arrowhead* without tail) (× 250).

After all 4 separation methods, laminin remained predominantly with the dermis; however, there were areas of patchy fluorescence seen at the polar aspect of some of the epidermal cells where the molecule was retained. This was more evident when the separation was accomplished by the induction of a blister (Fig 5*C*).

In contrast to the other macromolecules, after each of the DEJ separation procedures, BPA remained with the epidermis and was demonstrated as linear fluorescence along the polar aspects of the basal cells (Figs 5*A*, 6). BP serum left the corresponding dermal components unstained (Fig 5*A*). The intensity of BPA staining was markedly reduced in epidermis separated by warm saline. Therefore, semiquantitative studies on separated epidermis stained with BP serum were performed. BPA was readily demonstrated as a bright linear fluorescent band on the basal side of all of the separated epidermis specimens with a 1:320 or greater dilution of BP serum except when the skin was separated by warm saline. With warm saline separation of the skin, BPA was detectable with a 1:10 dilution of serum but was completely lost at 1:20.

By electron microscopy, the electron-dense basal lamina was found to remain with the dermis by all of the DEJ separation techniques as previously reported.

DISCUSSION

Based on electron microscopic studies, the DEJ of human skin has been divided into 4 components: (1) the plasma membrane of the palisading basal cells, (2) the electron-lucent lamina lucida, (3) the electron-dense lamina densa, and (4) a fibrillar area below the lamina densa containing anchoring fibrils and bundles of microfibril-like elements [23].

Ultrastructural studies have also shown that BPA and laminin are located almost exclusively within the lamina lucida area [16,24,25]. The electron-dense lamina densa appears to be rich in type IV collagen [18]. Heparan sulfate proteoglycan is

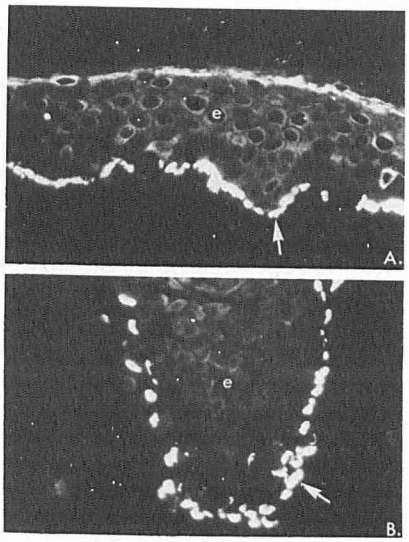

FIG 6. Epidermis (*e*) separated from dermis by cold 1 M salt (*A*) or cold trypsinization (*B*) and stained by IIF with BP sera diluted 1:320. *Arrows* point to retained BPA. (× 250).

thought to be localized in the lamina rara of the kidney [26] and above and below the lamina densa in skin (J.-M. Foidart et al, personal communication). The localization of type V collagen is less clear. According to Stenn, Madri and Roll [7], type V collagen is a basement membrane collagen, and its synthesis may be required for epidermal migration. Other investigators have found type V collagen in a pericellular localization around smooth muscle cells [8]. IIF staining of monkey esophagus sections with affinity-purified antibodies to type V collagen, in this study, showed the localization of type V collagen to be within the BMZ around blood vessels and between the epithelium and lamina propria (Fig 2). It was also localized around the fascicles of the esophageal smooth muscle bundles (Fig 2*B*). In the skin it was localized around dermal blood vessels and at the DEJ. The staining with type V collagen antibody could be blocked with type V collagen but not with laminin or type IV collagen. When the epidermis was separated from the dermis by any of the 4 methods, type V collagen within the BMZ clearly remained with the dermis.

In this study, adult human skin was separated at the DEJ by 4 different methods: cold trypsinization, cold 1 M salt, warm PBS, and by the induction of a blister with a suction device. These separation techniques are known to leave the lamina densa with the dermis and cleave the DEJ through the lamina lucida [6,10,11,23]. Since the lamina densa is thought to be rich in type IV collagen, one would expect that type IV collagen should be detected on the dermal side of the separation, and, indeed, with purified antibodies to type IV collagen, this was the case. This finding is consistent with the study of Saksela and colleagues [6] done on suction blisters. Epithelial cells are known to produce type IV collagen [27]. Keratinocytes have been shown to produce a lamina densa ultrastructurally [28, 29], but it is unclear whether or not they produce type IV collagen without dermal influences [30]. Even in the presence of the dermis, type IV collagen production beneath migrating epithelium is minimal or delayed [30–32]. However, it is still quite possible that type IV collagen is synthesized within keratinocytes and then extruded into the extracellular matrix where it contributes to the formation of the basement membrane.

Of the 3 known lamina lucida molecules—BPA, laminin, and heparan sulfate proteoglycan—this study demonstrates that BPA tends to remain with the epidermal cell after DEJ separation while laminin and the heparan sulfate proteoglycan remain with the dermis. BPA is, then, the basement membrane component most closely associated with the epidermal cell. When human keratinocyte suspensions are prepared by trypsinization, 20–50% of the cells will show polar staining by IIF with BP sera [12], while laminin, type IV collagen, and the heparan sulfate proteoglycan cannot be detected (unpublished observations). Further, there is some evidence that in vivo BPA is tenaciously associated with the basal cell and can be extracted off the cell only by harsh treatment or denaturing agents [33]. The antigen is more easily extracted from cultured cells ([34]. The fact that BPA is the basement membrane component most closely associated with the basal cell keratinocytes may relate to its function. Although the function(s) of BPA is unknown, this close association with the proliferative pool of keratinocytes would put it in position to act as a matrix molecule capable of influencing epidermal cell kinetics, migration, differentiation, and attachment.

The semiquantitative studies with BPA showed much less antigen on the epidermis after separation by warm PBS in comparison to the other techniques (Table I). Certain surface or intercellular glycoproteins on keratinocytes are sensitive to a thiol protease [35]. It may be that a similar protease acts on the BPA under the conditions of PBS at 37°C. If such a protease is responsible for the lack of BPA antigenicity in this situation, it could either degrade the molecule or release it from the basal cell, perhaps in a relatively native form.

When skin was separated by cold trypsinization, BPA clearly remained with the epidermis as noted with the other separation techniques. However, the staining pattern was slightly altered, with some separation occurring between individual basal cells. This "acantholysis" of the basal cells stained with BP sera produced a patchy but intense polar fluorescence (Fig 6*B*). Of the BMZ macromolecules tested, only the basement membrane heparan sulfate proteoglycan lost its antigenicity after cold trypsinization (Table I). Since the antibody is directed against the proteoglycan protein core [17], it may be that trypsin effectively degrades the molecule, making the antibody binding site unrecognizable.

The authors are grateful to Mr. Harold Shaefer for his technical assistance, to Drs. John Hassell and Hynda Kleinman for purified antibody to the basement membrane heparan sulfate proteoglycan, and to Drs. Stephen I. Katz, John R. Stanley, and George Martin for their thoughtful suggestions and review of the manuscript.

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0022-202X/83/8102-0153$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 81:153–157, 1983

Vol. 81, No. 2
Printed in U.S.A.

Presence of Basal Lamina-like Substance with Anchoring Fibrils Within the Amyloid Deposits of Primary Localized Cutaneous Amyloidosis

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The dermal-epidermal (DE) junction areas of skin specimens obtained from 16 patients with either lichen amyloidosis or macular amyloidosis were studied. In the dermal papillae where amyloid was deposited, elastic fibers frequently were absent, but periodic acid-Schiff reaction after diastase digestion was homogenously positive. Ultrastructural studies revealed that a basal lamina-like substance with anchoring fibrils was present between and within amyloid deposits. By indirect immunofluorescence technique using an anti-basement membrane zone antiserum obtained from a patient with bullous pemphigoid, specific linear fluorescence occurred at the DE junction, and in a reticular pattern in dermal papillae. It seemed that apoptotic keratinocytes of the epidermis brought down basal lamina and fine fibrous components attached to it when these cells dropped down to the papillary dermis and became the source of amyloid. These findings support the hypothesis that epidermal keratinocyte degeneration plays an important role in the histogenesis of cutaneous amyloidoses.

Manuscript received August 20, 1982; accepted for publication March 1, 1983

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Abbreviations:
BP-antibody: antiserum to the basement membrane zone of the skin
DE: dermal-epidermal
FITC: fluorescein isothiocyanate
O & G: orcein and Giemsa
PAS: periodic acid-Schiff
PBS: phosphate-buffered saline at pH 7.2

Filamentous degeneration of epidermal keratinocytes seems to play an important role in the histogenesis of lichen amyloi-