Differential Staining of Cytoid Bodies and Skin-Limited Amyloids With Monoclonal Anti-Keratin Antibodies

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The authors have used 5 different monoclonal antikeratin antibodies to study the antigenic profiles of cytoid bodies and skin-limited amyloids. Monoclonal antibodies AE1 (which stains the basal cell layer in normal human epidermis), AE2 (suprabasal layers), AE3 (whole epidermis), EKH4 (lower 2–3 layers), and EKH1 (recognizes all classes of intermediate filaments) were used to stain frozen skin sections by the indirect immunofluorescent or indirect immunoperoxidase technique. Cytoid bodies in lichen planus (LP) and discoid lupus erythematosus (DLE) were strongly stained with AE1, AE3, EKH4, and EKH1 antibodies but were negative with AE2. In contrast, amyloids in lichen amyloidosus and macular amyloidosis were stained strongly with EKH4 but only weakly or not at all with AE1, AE2, AE3, and EKH1. Amyloid associated with epithelial tumors showed closer immunologic profiles to cytoid body. These findings suggest that epidermal keratins are the major precursor substance of skin-limited amyloids as well as cytoid bodies in LP and DLE. Sequential changes in antigenic profiles from basal cells to amyloids through cytoid bodies further suggest that cytoid bodies may represent one of the precursor substances of skin-limited amyloids. (Am J Pathol 1984, 116:473–481)

IN RECENT YEARS, two types of abnormal skin deposits, ie, primary cutaneous amyloids and cytoid bodies (Civatte bodies, colloid bodies) have been thought to be of epidermal origin.[1–5] Among various types of amyloidoses, primary cutaneous amyloidosis and amyloidoses associated with cutaneous epithelial tumors are classified into a separate entity because amyloid deposition in the skin is not related to any systemic disease.[6–7] Amyloid deposition in these lesions is always limited to the subepidermal or tumor-adjacent area; sometimes, it can be found even within the epidermis or tumor parenchyma.[8–9] Sequential ultrastructural changes between epidermal keratinocytes and dermal amyloid have been reported.[10] Based on these findings, we have proposed a "filamentous degeneration theory" of tonofilaments to explain the pathogenesis of skin-limited amyloidoses.[4,5] We hypothesized that dermal amyloid might be keratin-related and derived from degenerated epidermal cells.[11,12] This possibility was supported by data as reported by us[12] as well as others[13,14] showing that immunohistochemically, polyclonal antibodies against epidermal keratin cross-react with this type of amyloid. In regard to cytoid bodies, both ultrastructural and immunologic data suggest that degenerated epidermal cells contribute to their formation.[2,4,5,15–22] It has also been suggested that cytoid bodies, at least in part, may be precursors of amyloids.[5,13,22–24] In the present study, we used a panel of 5 different monoclonal antibodies to further study the immunologic characteristics of skin amyloids and cytoid bodies.

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Table 1—Specificities of Monoclonal Antibodies

Monoclonal antibodies	Antigen specificity	Staining of normal human epidermis
AE1	Keratin (50, 56 kd)*	Basal cell layer
AE2	Keratin (50, 58, 65-67 kd)	Suprabasal layers
AE3	Keratin (52, 58, 65-67 kd)	Whole layers
EKH4	Keratin (50 kd)	Lower 2-3 layers
EKH1	All classes of intermediate filaments (keratin, vimentin, desmin, GFAP, neurofilaments)	Whole layers

* Because of the masking of antigen *in situ*, all of these keratin species are not stained.

Materials and Methods

Tissue Specimens

Amyloid-positive skin biopsies were obtained from 2 patients with lichen amyloidosus, a patient with macular amyloidosis, 2 patients with basal cell epithelioma (BCE), and 3 patients with nodular amyloidosis. Specimens of skin and heart tissues from a patient with primary systemic amyloidosis were obtained at autopsy. Serial sections of each tissue were stained with Congo red and crystal violet for amyloid identification. In addition, 5 skin biopsy specimens with cytoid bodies were obtained from 3 patients with discoid lupus erythematosus (DLE) and 2 patients with lichen planus (LP). The tissue specimens were embedded in O.C.T. Compound (Lab-Tek Products, Naperville, Ill), snap-frozen in liquid nitrogen, and stored in −40 C until use.

Monoclonal Antibodies

Five monoclonal antibodies, AE1, AE2, AE3, EKH4, and EKH1, were used in this study. Preparation of these antibodies was described elsewhere.[25-28] Briefly, BALB/c mice were immunized with either extracted epidermal keratins (AE1, AE2, AE3)[25,26] or the human trichilemmoma cell line (EKH4,[27] EKH1[28]) and the spleen cells from immunized mice were fused with mouse myeloma cell line P3x63Ag8 using the hybridoma technique.[29] The trichilemmoma cell line[30] was used as an immunogen because it produces keratins in its cytoplasm.[31] AE1, AE2, AE3, and EKH4 are anti-keratin antibodies with different specificities, as shown in Table 1. EKH1 antibody reacts with all classes of intermediate filaments, including keratin, vimentin, desmin, glial fibrillary acidic protein (GFAP), and neurofilaments.[28]

Immunohistochemistry

Frozen tissue specimens were cut into 4-μ sections in a cryostat and air-dried. For the indirect immunofluorescent technique, primary incubation (30 minutes at room temperature) was done with either 1:50-diluted mouse ascites fluid or undiluted tissue culture supernate of the hybridomas. Secondary incubation was carried out with 1:20-diluted FITC-conjugated goat anti-mouse IgG serum (Cappel Laboratories, Cochranville, Pa) for 30 minutes at room temperature. After each incubation, the sections were rinsed twice in phosphate-buffered saline (PBS) for 10 minutes each, then mounted with paraphenylene-diamine (Sigma Chemical Co., St. Louis, Mo) mounting buffer[32] and observed under a Zeiss fluorescence microscope. In some experiments, the cryostat sections were pretreated with various chemicals for 10 minutes at room temperature, including 5% acetic acid in 70% ethanol, or with 8 M urea and 25 mM 2-mercaptoethanol in Tris buffer. For control studies, primary antibodies were substituted by control ascites or PBS. For immunoelectron microscopy using indirect immunoperoxidase technique, the sections were pretreated with 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity.[33] The secondary antibody was 1:10-diluted peroxidase-conjugated goat anti-mouse IgG antibody (TAGO, Inc., Burlingame, Calif). After diaminobenzidine (Sigma) reaction in the presence of hydrogen peroxide,[34] the sections were fixed with 5% glutaraldehyde, followed by 1% osmium tetroxide, and embedded in Araldite. Ultrathin sections were observed with or

Table 2—Reactivities of Amyloids and Cytoid Bodies With Monoclonal Antibodies

	AE1	AE2	AE3	EKH4	EKH1
A. Cytoid bodies					
Discoid lupus erythematosus					
1	+	−	+	+ +	+
2	+	−	+ +	+ +	+
3	+	−	+	+ +	+
Lichen planus					
1	+ +	−	+ +	+ +	+
2	+	−	+ +	+ +	+
B. Amyloids					
Lichen amyloidosus					
1	−	−	−	+ +	−
2	−	−	±	+ +	−
Macular amyloidosis	−	−	±	+ +	−
Basal cell epithelioma					
1	−	−	±	+ +	±
2	+	−	+	+ +	+
Nodular amyloidosis					
1	−	ND	ND	−	ND
2	−	−	−	−	−
3	−	−	−	−	−
Primary systemic amyloidosis					
Skin lesion	−	−	−	−	−
Heart lesion	−	−	−	−	−

+ +, strong staining; +, moderate staining; ±, weak staining; −, negative; ND, not done.

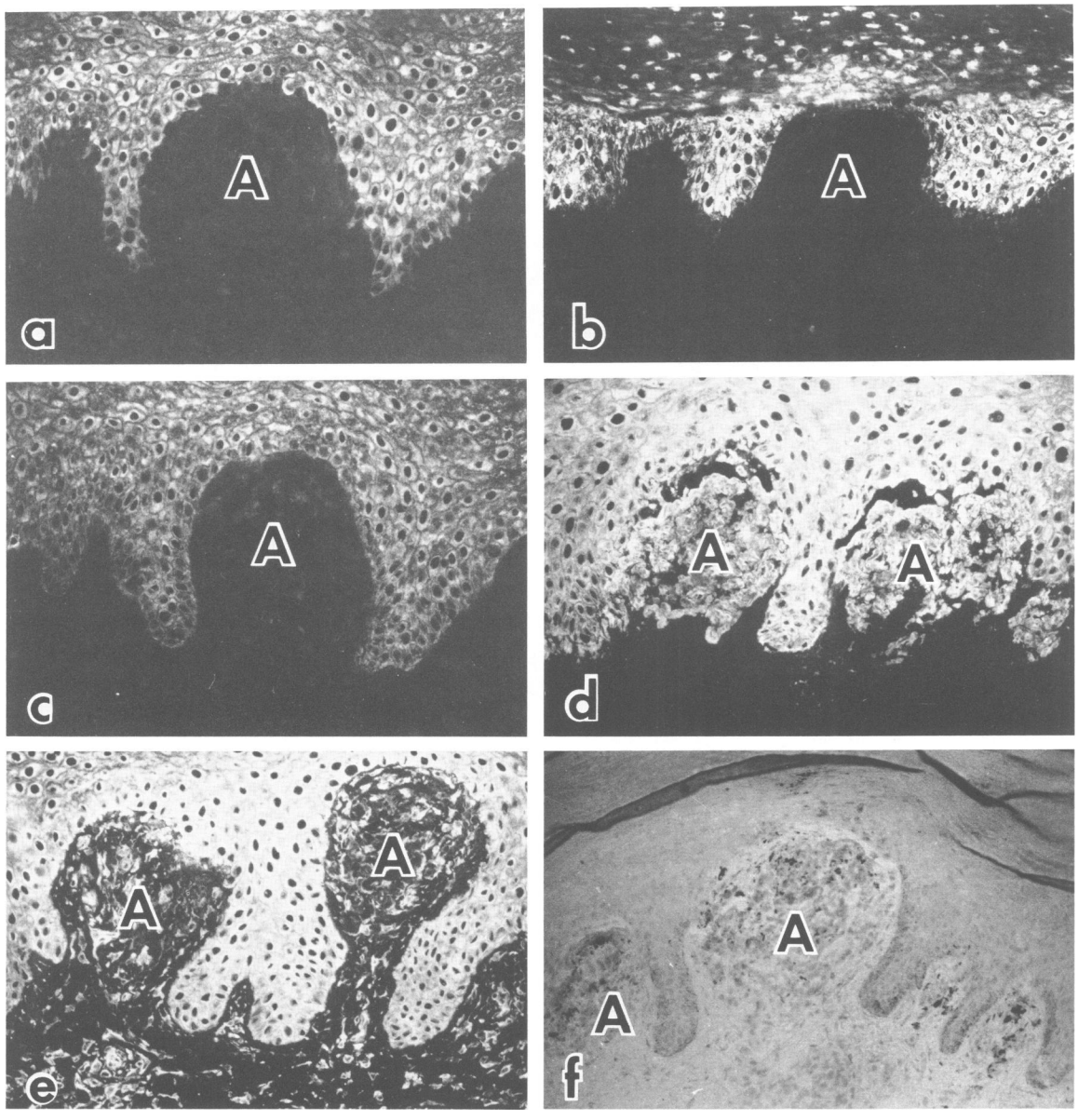

Figure 1 — Lichen amyloidosus. **a–e** — Indirect immunofluorescence staining with monoclonal antikeratin antibodies. **a** — AE1 does not stain amyloid (*A*). Positive staining can be seen in most of the epidermis. Basal cells are stained only weakly. (× 250) **b** — AE2 does not react with amyloid. Suprabasal layers of the epidermis is stained positively. (× 250) **c** — AE3 does not react with amyloid. Whole epidermis shows positive staining. (× 250) **d** — EKH4 strongly stains amyloid deposition in dermal papillae. Whole epidermis is also positively stained, instead of the usual pattern of staining with EKH4, ie, basal cell layer predominance. (× 250) **e** — EKH1 stains whole epidermis and dermal cells, including fibroblasts (which contain vimentin). There is no positive reaction on amyloid deposition. Notice the many fibroblasts within the amyloid mass. (× 250) **f** — Crystal violet staining shows purplish violet color in dermal papillae, confirming amyloid deposition in the same specimen. (× 250)

without electron stain. For control studies, primary antibodies were omitted or substituted for by control ascites and were processed in the same manner.

Results

Immunofluorescence Studies

Immunohistochemical staining properties of amyloids and cytoid bodies varied, depending on the monoclonal antibodies (Table 2). Among the 5 monoclonal antibodies used in this study, EKH4 consistently stained amyloids in primary cutaneous amyloidosis as well as BCE-associated amyloid. Other antibodies failed to react with amyloids in primary cutaneous amyloidoses except for a weak reaction of AE3 antibody. In a case of lichen amyloidosus, amyloid deposition located in papillary dermis was strongly stained with EKH4; whereas AE1, 2, 3, and EKH1 did not react with it (Figure 1a–f). In the

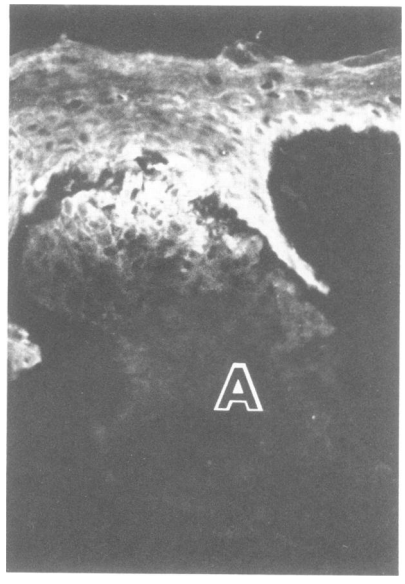

Figure 2—Macular amyloidosis, indirect immunofluorescence staining. AE3 recognizes the upper part of amyloid deposition (*A*) which is adjacent to or continuous with the epidermis. Notice that the lower part of amyloid deposition is not recognized by AE3. In the epidermis, whole layers are stained. (×250)

overlying epidermis, the entire epithelium was stained with EKH4, instead of a basal cell predominant pattern, probably because of abnormal keratinization.[26,27] Crystal violet staining of a neighboring section confirmed the amyloid nature of the EKH4-positive material in the same area of papillary dermis (Figure 1f). In macular amyloidosis, amyloid deposition in papillary dermis was also reacted with EKH4; whereas AE1, 2, 3, and EKH1 reacted negatively or only weakly. In tissue sections stained with AE3, the upper portion of amyloid deposition which was adjacent to overlying epidermis showed positive staining, and the intensity is decreased when the amyloid deposit is deeper, away from the epidermis (Figure 2). In the cases of BCE-associated amyloid, EKH4 strongly recognized amyloid (Figure 3a). In addition, AE3 and EKH1 weakly stained the amyloid in the Case 1; and AE1, AE3 (Figure 3b) and EKH1 moderately stained the amyloid in the Case 2. The presence of amyloid was confirmed by positive staining of serial sections with Congo red and crystal violet.

In a case of systemic amyloidosis, both skin and heart amyloid failed to react with any of 5 monoclonal antibodies. Amyloid of nodular amyloidosis did not react with any antibodies tested (Figure 4). In all sections of DLE and LP, cytoid bodies were positively stained with AE1, AE3, EKH4, and EKH1 antibodies (Figure 5). In DLE cases, positively stained cytoid bodies were found even in deep dermis.

Immunoelectron Microscopy

Indirect immunoperoxidase staining of amyloid deposition of macular amyloidosis with EKH4 antibody revealed electron-dense reaction products located on the amyloid islands (Figure 6). In DLE cases, EKH4-positive staining was seen on cytoid bodies (Figure 7). In both cases, the sections were observed without electron staining. The presence of amyloid and cytoid bodies was confirmed on the same sections or serial sections after electron staining; thin, straight filaments in amyloid islands and closely packed, wavy filaments in cytoid bodies were observed.

Chemical Treatment of Amyloid Sections

In order to enhance the selective staining of amyloid or to suppress epidermal reactivity with EKH4 antibody, amyloid sections were pretreated with different reagents before EKH4 staining. The result is shown in Table 3. In the fixative group, 5% acetic acid in 70% alcohol gave good results. That is, epidermal

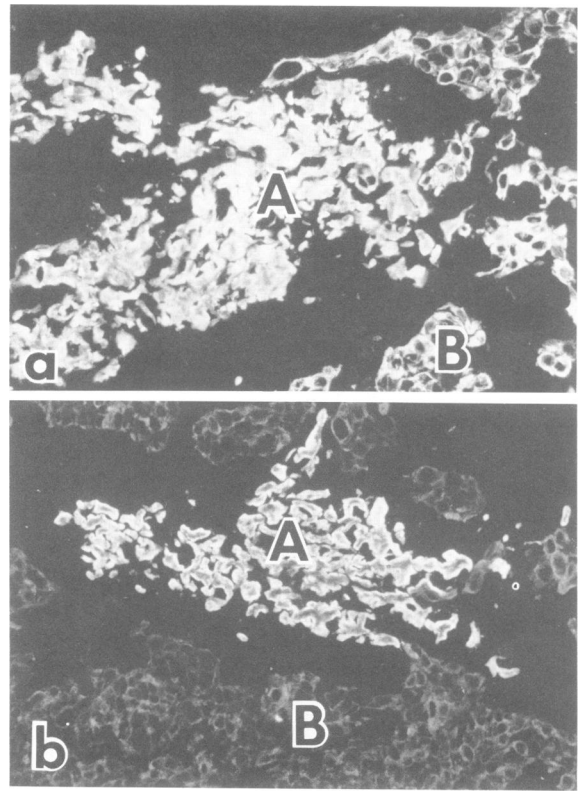

Figure 3—Basal cell epithelioma, Case 2. Indirect immunofluorescence staining. **a**—EKH4 staining shows positive reaction on BCE tumor cells (*B*) and amyloid (*A*). No stromal cell is stained. (×250) **b**—AE3 recognizes amyloid (*A*) moderately, but it recognizes BCE tumor cells (*B*) weakly. No staining in stroma. (×250)

reactivity was depressed, whereas amyloid remained strongly positive with EKH4 antibody (Figure 8a). The best result was obtained in the sections treated with 8 M urea and 25 mM 2-mercaptoethanol, in which epidermal keratin was almost completely extracted, whereas amyloid remained insoluble and immunologically unaltered, to give strong reactivity with EKH4 antibody (Figure 8b).

Discussion

Although various types of amyloids show similar tinctorial and ultrastructural features, recent progress in amyloid research has made it possible to further classify amyloids into several different groups of proteins.[35] As for amyloids of primary cutaneous amyloidosis and epithelial tumor-associated amyloid, it was suggested that they are derived from epidermal keratin (tonofilament) of keratinocytes based on ultrastructural and immunologic studies.[5,10-14] Recently, we[12] as well as others[13] have shown that polyclonal antikeratin antibodies recognize these amyloids, suggesting that they are immunologically related to epidermal keratin. Since there are several different keratins in epidermis,[25] it was our interest to study skin limited amyloids with specific monoclonal antikeratin antibodies. The present study revealed that out of 5 monoclonal antibodies, only one antibody, EKH4, constantly and strongly labeled amyloids in primary cutaneous amyloidoses as well as in all cases of BCE-associated amyloid. Other antibodies did not react with amyloids in lichen amyloidosus or macular amyloidosis, although AE1, AE3, and EKH1 also reacted with BCE-associated amyloid. This result indicates that there exists at least one common antigenic determinant on keratins and on skin amyloids which is selectively recognized by EKH4. In other words, during amyloid formation some part of the keratin molecules are altered or lost, but the epitope on which EKH4 antigenic determinant is located remains unchanged. The other explanation, that the certain keratin subset which is recognized only by EKH4 and not by the others forms amyloids, is not likely because such a keratin subset does not exist; the combination of AE1, 2, and 3 recognizes all major keratin polypeptides in the epidermis.[36]

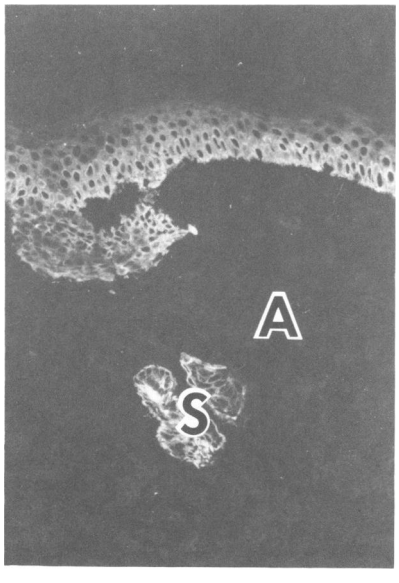

Figure 4 — Nodular amyloidosis of the skin. Indirect immunofluorescence staining with EKH4 antibody shows positive reaction in lower epidermis and sebaceous gland (*S*). Amyloid (*A*) is negatively stained. (× 160)

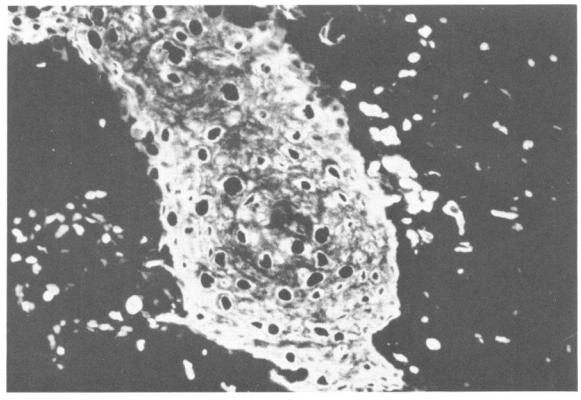

Figure 5 — Cytoid bodies in DLE. Indirect immunofluorescence stainings. AE1 recognizes keratinocytes in the epidermis and numerous cytoid bodies in dermis. (× 250)

None of the antibodies recognized amyloids of systemic amyloidosis or nodular amyloidosis of the skin. These results confirm our previous report using polyclonal antikeratin antibody.[12]

Several reports suggested that cytoid bodies could be precursors of amyloids. Ultrastructurally, sequential changes from PUVA-damaged keratinocytes of psoriatic skin through cytoid bodies to amyloid formation have been reported.[5] To further investigate this point, we also studied the immunologic aspects of cytoid bodies. The present study showed that cytoid bodies are recognized by AE1, AE3, EKH4, and EKH1 antibodies. Since this pattern of reactivity is identical to that of epidermal basal cells (Figure 9), cytoid bodies may be derived from degenerated epidermal basal cells or dedifferentiated keratinocytes, which are similar to basal cells. In the epidermis overlying the amyloid lesion, keratinocytes in upper layers were also recognized by EKH4 or AE1 antibodies (Figure 1a and d); these keratinocytes could be the

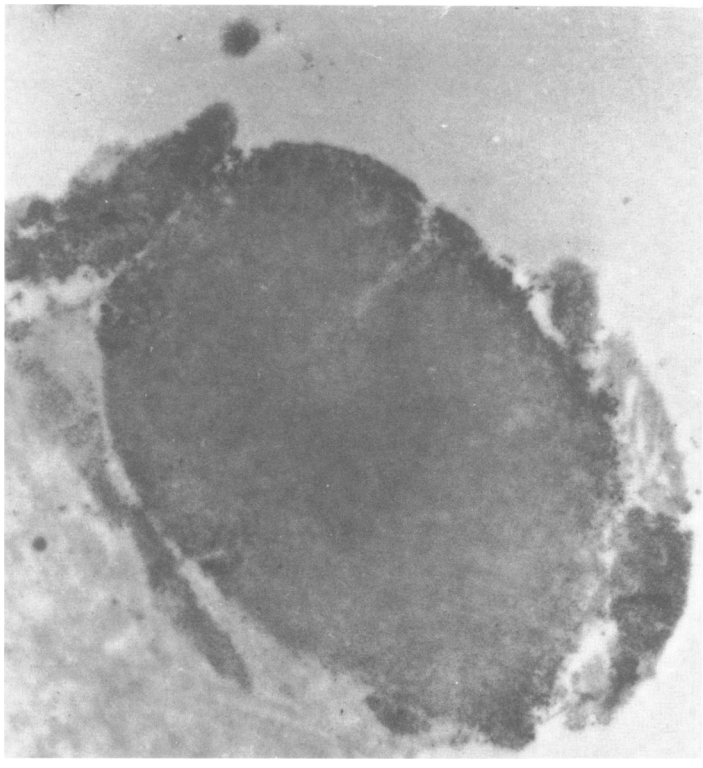

Figure 6—Immunoelectron microscopy of macular amyloidosis with EKH4 staining. Low magnification picture shows electron-dense reaction products on amyloid deposition. No electron stain. (×12,800)

source of "amyloid-prone" cytoid bodies. In the proliferative skin disorders, such as psoriasis, actinic keratosis, seborrheic keratosis, or inflammatory conditions, keratinocytes in the upper layers of the epidermis also were positive for both AE1 and EKH4 antibodies.[26,27]

Gomes et al[21] showed that cytoid bodies were positively recognized by suprabasal layers specific for polyclonal antikeratin antisera (62k, 67k specific). We have just shown that cytoid bodies could be labeled with basal layer antibodies such as AE1 and EKH4. This discrepancy might be due to a difference of

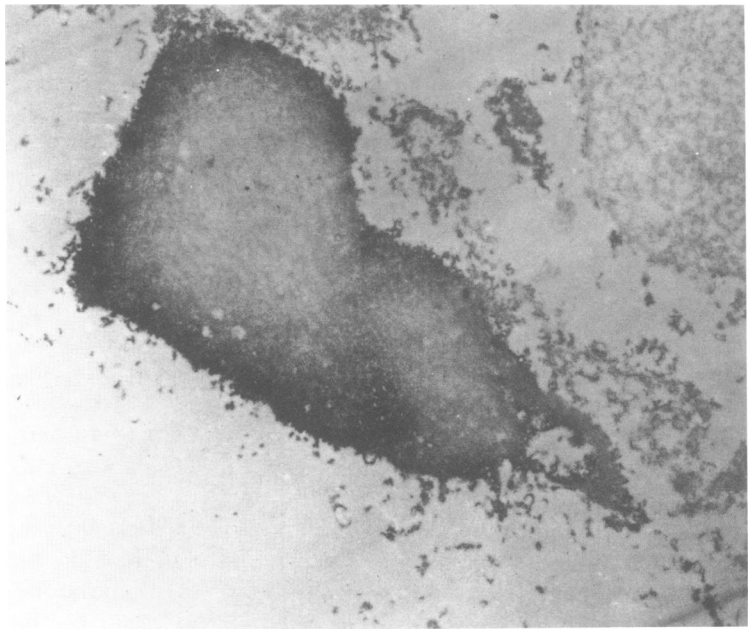

Figure 7—Immunoelectron microscopy of a cytoid body in DLE. EKH4 staining shows electron-dense reaction products on a cytoid body in dermis predominantly along the periphery. No electron stain. (×12,800)

Table 3—Reactivities of Epidermis and Amyloid in Lichen Amyloidosus With EKH4 After Chemical Treatment

	Epidermis	Amyloid
Before treatment	+ +	+ +
70% alcohol	+	+ +
Acetone-alcohol (1:1)	+ +	+ +
5% acetic acid-alcohol	±	+ +
0.05% trypsin	+ +	+ +
8 M urea	+	+ +
8 M urea + 2-mercaptoethanol	–	+ +

+ +, strong staining; +, moderate staining; ±, weak staining; –, no staining.

specificities and characteristics between monoclonal and polyclonal antibodies. The immunohistochemical localization of keratin antigens in different layers of normal epidermis, cytoid bodies, and amyloids is schematically summarized in Figure 9. The result clearly indicates that there are sequential changes from epidermal basal cells through cytoid bodies to amyloids. It is interesting that BCE-associated amyloid showed its antigen property almost identical to cytoid bodies. We speculate that in BCE-associated amyloid, the antigenic profile remains minimally changed from its precursor substance, ie, cytoid bodies or basaloid cells.

In macular amyloidosis, we observed an interesting phenomenon. In the upper part of the amyloid mass adjacent to the epidermis, AE3 antibody recognized the amyloid, and the staining intensity of amyloid was decreased as it was located deeper and more removed from the epidermis. It finally became negative within the same amyloid mass (Figure 2). Although there was no difference within the amyloid mass as to ultrastructural and histochemical characteristics, such as Congo red or crystal violet staining, amyloid deposition adjacent to epidermis seems to be newly formed and hence AE3-positive. Another interesting finding was obtained with EKH1 antibody. Since EKH1 recognizes all classes of intermediate filaments,

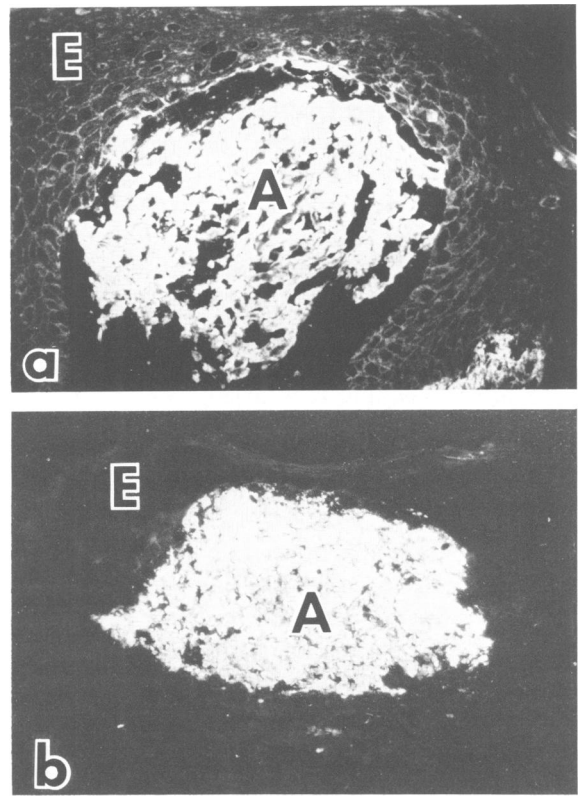

Figure 8—Lichen amyloidosis. Indirect immunofluorescence staining with EKH4 after pretreatment with various chemicals. **a**—After pretreatment with 5% acetic acid in ethanol for 15 minutes. Epidermal reactivity is greatly reduced, whereas amyloid remains positive. (×250) **b**—Pretreated with 8 M urea and 25 mM 2-mercaptoethanol for 15 minutes. Epidermis (*E*) shows no staining, whereas amyloid (*A*) remains positive. Notice selective staining on amyloid deposition. (×205)

we expected that EKH1 would react with amyloids. However, EKH1 decorated cytoid bodies regularly and BCE-associated amyloid in one case and failed to do so in cases of amyloids. Negative staining of amyloids with EKH1 suggests that the antigenic characteristics of intermediate filaments are shared in cytoid

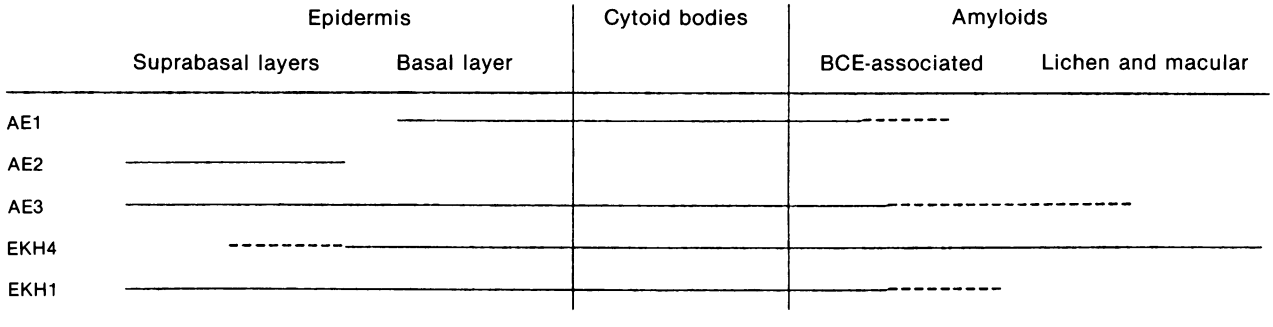

Figure 9—Expression of different antigens in epidermis, cytoid bodies, and skin amyloids recognized by monoclonal antibodies.

bodies and some early stage amyloids, such as BCE-associated amyloid but completely lost in fully developed amyloid filaments in primary cutaneous amyloids. This antigenic alteration may be related to β-transformation of α-keratin polypeptides during amyloid formation, because EKH1 recognizes all α-intermediate filaments so far tested.[28] In addition, EKH1 antibody visualized numerous fibroblasts (containing vimentin) within amyloid deposition. The role of fibroblasts in amyloid formation still remains unclear. If vimentin has anything to do with cutaneous amyloid, the most logical variety would be nodular amyloidosis. Elastofibrils of degenerated elastic fiber should also be considered as the source of this variety, because recent works of Breathnach et al[37] demonstrated amyloid P component in this variety of cutaneous amyloidosis.

Although antibodies to certain types of amyloid proteins have been reported,[38,39] attempts to produce skin amyloid-specific antibody has been unsuccessful because of the difficulty of purifying amyloid from the skin lesion. In the present study, we found that pretreatment of amyloid tissue sections with acid-alcohol or 8 M urea plus 2-mercaptoethanol reduces or eliminates the EKH4 reactivity of the epidermis without altering the amyloid staining. In the purification of skin amyloid from the lesion by affinity chromatography, for example, these reagents will be useful in inhibiting or eliminating epidermal keratin.

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