EFP and HFP protein than previously has been appreciated. They both occur first as citrate-soluble reduced proteins and consist of a number of polypeptides in the same M_r range. They have different amino acid compositions, the HFP being much richer in cystine. It is possible, however, that the helical regions of the molecules are similar and the difference resides in the nonhelical segments which make up an appreciable part of the molecule. Isolation of individual chains and amino acid sequences are necessary to establish this.

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The Use of Monoclonal Antibody to Keratin in Human Epidermal Disease: Alterations in Immunohistochemical Staining Pattern

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A monoclonal antikeratin antibody, designated AE1, was used to stain frozen sections of normal and abnormal human skin by the immunofluorescence and peroxidase-antiperoxidase techniques. In normal human epidermis and ichthyosis vulgaris, a nonproliferative epidermal disease, this antibody selectively stained epidermal basal cells. Very different staining patterns were observed in various other epidermal diseases. A suprabasal staining pattern was observed in psoriasis (16 cases), verruca (9), seborrheic keratosis (5), actinic keratosis (2), as well as the epidermis adjacent to certain epidermal neoplasms (4). Basal cell carcinoma (7) showed weak, homogeneous staining. In contrast, a disorganized pattern consisting of cells with various staining intens-

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Abbreviations:
AK: actinic keratosis(es)
BCC: basal cell carcinoma(s)
BD: Bowen's disease
H-E: hematoxylin and eosin
IF: immunofluorescence
PAP: peroxidaseantiperoxidase
PBS: phosphate-buffered saline
SCC: squamous cell carcinoma(s)
SK: seborrheic keratosis(es)

ities was observed in Bowen's disease (2) and squamous cell carcinoma (4). Although the biochemical basis for these altered staining patterns remains to be elucidated, these results provide further evidence that epidermal keratin expression can be affected by various disease states. Moreover, our data suggest that a common alteration in keratin expression, as defined by the suprabasal AE1 staining pattern, exists in psoriasis and a number of other benign hyperproliferative epidermal diseases.

Keratinocytes in normal human epidermis undergo an orderly pattern of maturation during their migration from the basal layer through the spinous and granular layers to the cornified layer. This progression is accompanied by systematic changes in the synthesis of keratins [1–8] which are the water-insoluble protein subunits of tonofilaments.

Recent evidence indicates that keratin composition is a sensitive marker for epidermal differentiation. For example, the keratin composition of cultured human epidermal cells differs significantly from that of in vivo epidermis [9–12]. In addition, the expression of different keratins can be correlated with different stages of epidermal development [13–15], and with types of epithelial differentiation [2,16–25]. Finally, some alterations in keratin expression have been described in epidermal diseases including psoriasis, verrucae, and epidermal neoplasms [26–35].

Immunologic analyses of keratins have provided valuable information concerning the tissue-distribution of keratins [36–39] and the expression of keratin antigens during normal and abnormal keratinization [3–7,32,33]. However, characterization of keratins in disease has thus far been limited to the use of conventional antisera, many of them prepared against keratins isolated from the stratum corneum. Due to the fact that keratins undergo partial proteolysis during the final stages of keratinization [1,2,6,7], individual keratins isolated from the stratum corneum are frequently contaminated by degradative products of the higher M_r components; antisera raised against such keratin mixtures are therefore frequently not well defined.

We have developed several monoclonal antibodies to human keratins using the hybridoma technique [6]. By immunofluorescent (IF) and peroxidase-antiperoxidase (PAP) staining techniques, one of these antibodies, designated AE1, has been shown to stain predominantly basal cells in frozen sections of normal human epidermis [6]. The specificity of this antibody for basal cells, which play a central role in initiating the sequence of epidermal differentiation, makes it potentially useful for the analysis of normal and pathologic keratinization. We demonstrate here that the AE1 staining pattern was indeed markedly altered by various epidermal disease processes. Furthermore, we show that a "suprabasal" staining pattern (instead of the normal "basal" staining pattern) was common to a large number of benign hyperproliferative epidermal diseases including psoriasis and verrucae. The same suprabasal staining pattern was observed in cultured normal human epidermal keratinocyte colonies, indicating that the suprabasal AE1 staining pattern cannot be disease-specific and may be related to a hyperproliferative state of keratinocytes.

MATERIALS AND METHODS

Antibody Preparation

Balb/C mice were immunized with sodium dodecyl sulfate-denatured human epidermal keratins [6]. Spleen cells from these immunized mice were hybridized with P3 × 63 Ag8 (abbreviated P3) myeloma cells using the hybridoma technique [40]. Hybridoma supernatants were assayed by an ELISA method [6] and subsequent cloning of positive wells was done in soft agar [41].

Antibody Characterization

Supernatant fluid from a repeatedly cloned hybridoma cell line AE1 (abbreviation of "anti-epithelial component one") was used as the source of the antibody. This antibody has been shown to be highly specific for keratins according to the following criteria. First, it selectively stains keratin fibers in cultured human epidermal cells and a variety of other epithelial cells. Second, immunofluorescent staining of frozen sections of various tissues showed specific binding of AE1 antibody to epithelia, with no detectable staining of any nonepithelial cell types. Finally, immunoblot analysis showed that AE1 antibody reacts with 2 keratins (M_r 50,500 and 56,500), but not with any other proteins of human epidermis [6,22].

Patient Selection

Normal breast, abdomen, face, and leg skin samples obtained from surgical specimens or autopsy were used as controls. A sample of ichthyosis vulgaris was obtained by shave biopsy. From 16 patients with clinically active psoriasis, diagnostic 4-mm punch biopsies of lesional skin were obtained; 12 had typical psoriatic plaques, 3 had guttate lesions, and 1 had an erythroderma. Three patients with psoriasis consented to a 2-mm biopsy of perilesional uninvolved skin 4 mm away from an active lesion.

Verrucae were obtained by shave biopsy or curettage in the course of treatment and these included 2 condyloma accuminata, 1 plantar wart, 3 filiform warts, and 3 common warts. In the same fashion 5 seborrheic keratoses (SK), 2 actinic keratoses (AK), 2 samples of Bowen's disease (BD), and 7 basal cell carcinomas (BCC) were obtained.

Four biopsy-proved invasive squamous cell carcinomas (SCC) were removed by elliptical excision under local anesthesia. All samples were flash-frozen in liquid nitrogen and stored at −70°C prior to sectioning and staining.

Cultured Human Epidermal Cells

Newborn human foreskin epidermal cells were grown in Dulbecco's minimal essential medium containing 20% fetal calf serum, hydrocortisone (0.5 μg/ml), and epidermal growth factor (15 ng/ml) in the presence of lethally irradiated (4000 rad) 3T3 feeder cells as described by Rheinwald and Green [42]. Vertical frozen sections of cultured human epidermal colonies (5 days postconfluency) were prepared according to Green et al [43].

Immunofluorescent Staining

Frozen sections (8 μm) of tissues and cultured epidermal colonies were air-dried, hydrated in phosphate-buffered saline (PBS), incubated with AE1 conditioned medium (37°C for 1 h), washed in PBS, incubated with fluorescein isothiocyanate-conjugated rabbit antimouse IgG (37°C for 30 min), washed in PBS, and mounted in Gelvatol [36,37].

Peroxidase-Antiperoxidase (PAP) Staining

Frozen tissue sections (8 μm) were also stained with AE1 antibody using the PAP technique [44]. Immunochemicals used included mouse PAP (Sternberger-Meyer Immunocytochemical, Jarrettsville, Maryland), 3,3-diaminobenzidine tetrahydrochloride (Sigma, St. Louis, Missouri), and goat antimouse IgG (Miles Laboratories, Elkhart, Indiana). All sections were washed in 1% hydrogen peroxide prior to staining to eliminate endogenous peroxidase activity. Controls consisted of substitution of the monoclonal antibody by culture medium conditioned by P3 myeloma parent cells and mouse preimmune serum; neither demonstrated any staining activity. In all cases examined, IF and PAP staining generated similar results.

Hematoxylin and Eosin (H-E) Staining

Portions of all specimens were fixed in formalin, paraffin-embedded, sectioned, and stained with routine H-E. In addition, serial frozen sections adjacent to those stained with the monoclonal antibody were stained by H-E in order to correlate the histologic features with the immunohistochemical staining data.

RESULTS

Controls

Using the IF technique, 20 samples of normal skin from a variety of body sites (except palm and sole) demonstrated basal layer staining (Fig 1*a*) [6]. Although the staining intensity by the IF technique varied somewhat depending on the age of the donor and the freshness of the sample [6], by the PAP technique all samples demonstrated intense and uniform staining of basal cells and, occasionally, a few cells immediately above the basal layer (Fig 1*b*). Uninvolved (perilesional) skin of 3

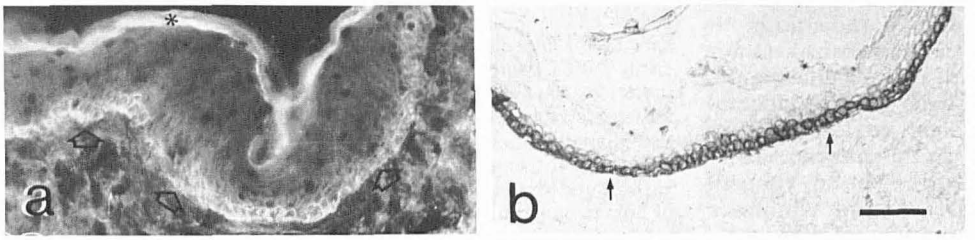

FIG 1. Immunohistochemical staining of normal human epidermis by AEl monoclonal antikeratin antibody. *a*, Normal abdominal skin, IF. *Arrows* indicate dermal-epidermal junction. *Asterisk* denotes the nonspecific staining of the stratum corneum [6,36]. *b*, Same specimen, PAP. Note the intense staining of basal layer. (*a*) and (*b*) are of the same magnification; *scale bar* in (*b*) = 50 μm.

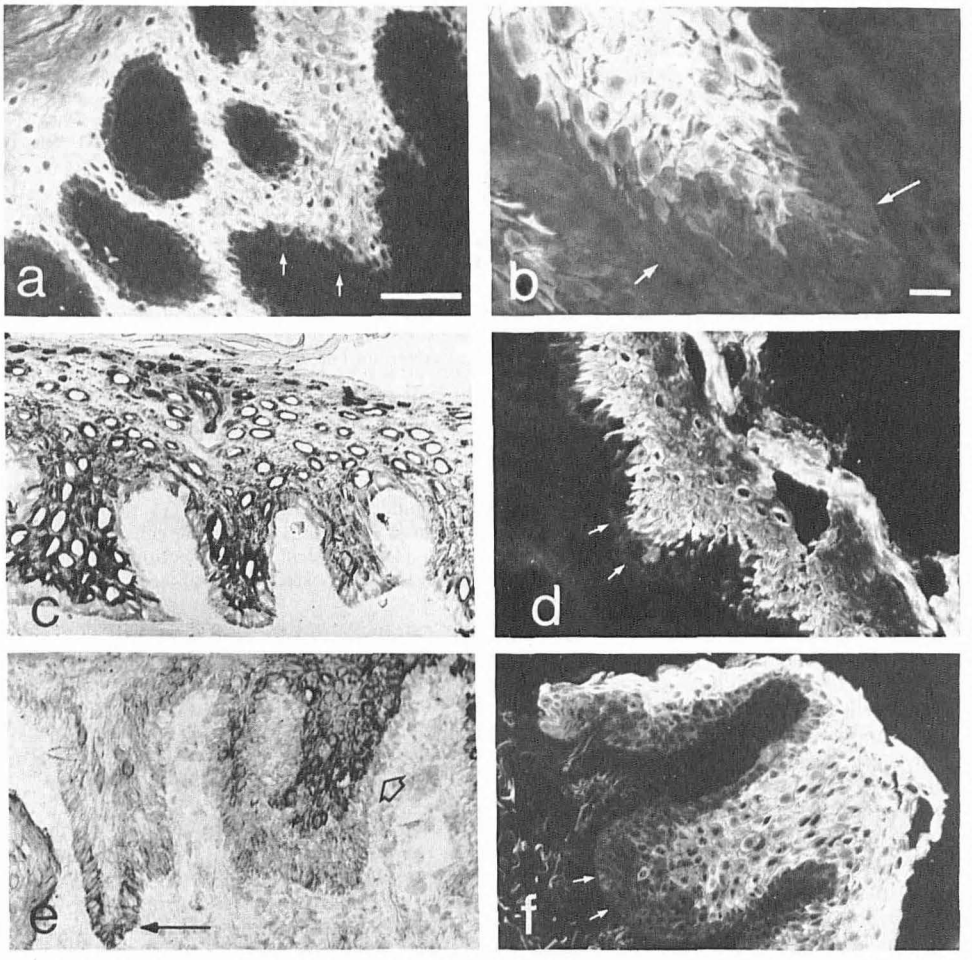

FIG 2. Immunohistochemical staining of psoriasis by AEl antibody. *a*, Base of a psoriatic plaque showing intense suprabasal staining (IF). *Arrows in all IF pictures* denote the dermal-epidermal junction. *b*, A higher magnification of a rete ridge within a plaque (IF). Although not shown here, a perilesional, uninvolved skin specimen (4 mm away from the edge of this lesion) demonstrated normal basal layer staining (IF). *c*, PAP staining of a psoriatic plaque, confirming the suprabasal staining pattern. *d*, A psoriatic erythroderm lesion showing suprabasal staining. *e*, PAP staining of a guttate lesion. Note the basal staining pattern in one rete ridge (*arrow*) and the suprabasal staining (tangential cut) in another rete ridge (*large arrowhead*). *f*, A guttate lesion showing suprabasal staining (IF). All pictures except (*b*) are of the same magnification. *Scale bar* in (*a*) = 50 μm; in (*b*) = 10 μm.

psoriatic patients and a specimen of ichthyosis vulgaris demonstrated the normal basal staining pattern (not shown).

Psoriasis

When AEl was used to stain frozen sections of psoriatic skin, a pattern dramatically different from that of normal skin was observed. In plaque lesions, epidermal cells above the basal layer demonstrated intense staining, while the basal cells reacted poorly with the antibody (Fig 2*a*,*b*, IF results; Fig 2*c*, PAP). The term "suprabasal staining" was used to describe this abnormal staining pattern. A similar staining pattern was observed in the erythrodermic lesion (Fig 2*d*). In the 3 guttate lesions examined so far, a few rete ridges within a single lesion demonstrated the normal basal staining pattern (Fig 2*e*); however, the predominant staining pattern was again suprabasal (Fig 2*e*,*f*).

Verrucae

Nine different verrucae representing 4 histologic types (condyloma, plantar, filiform, and common) were examined. All were found to stain by AEl antibody in a suprabasal fashion (Fig 3*a*–*d*) almost indistinguishable from that seen in psoriasis.

Seborrheic Keratosis

Five SK, 4 of the acanthotic type and 1 irritated SK lesion, were studied. Both the acanthotic and irritated SK exhibited suprabasal staining (Fig 3*e*) identical to that seen in psoriasis and verrucae.

Actinic Keratosis

The AK demonstrated the suprabasal pattern (Fig 4*a*). However, unlike the benign disorders described above, not all cells in AK were stained with equal intensity (Fig. 4*b*). The heterogeneity of staining intensity was relatively minimal, however, when compared with that seen in malignant tumors (see below).

Bowen's Disease

A highly disorganized staining pattern consisting of a mixture of intensely and weakly stained cells (Fig 5*a*,*b*) was observed in

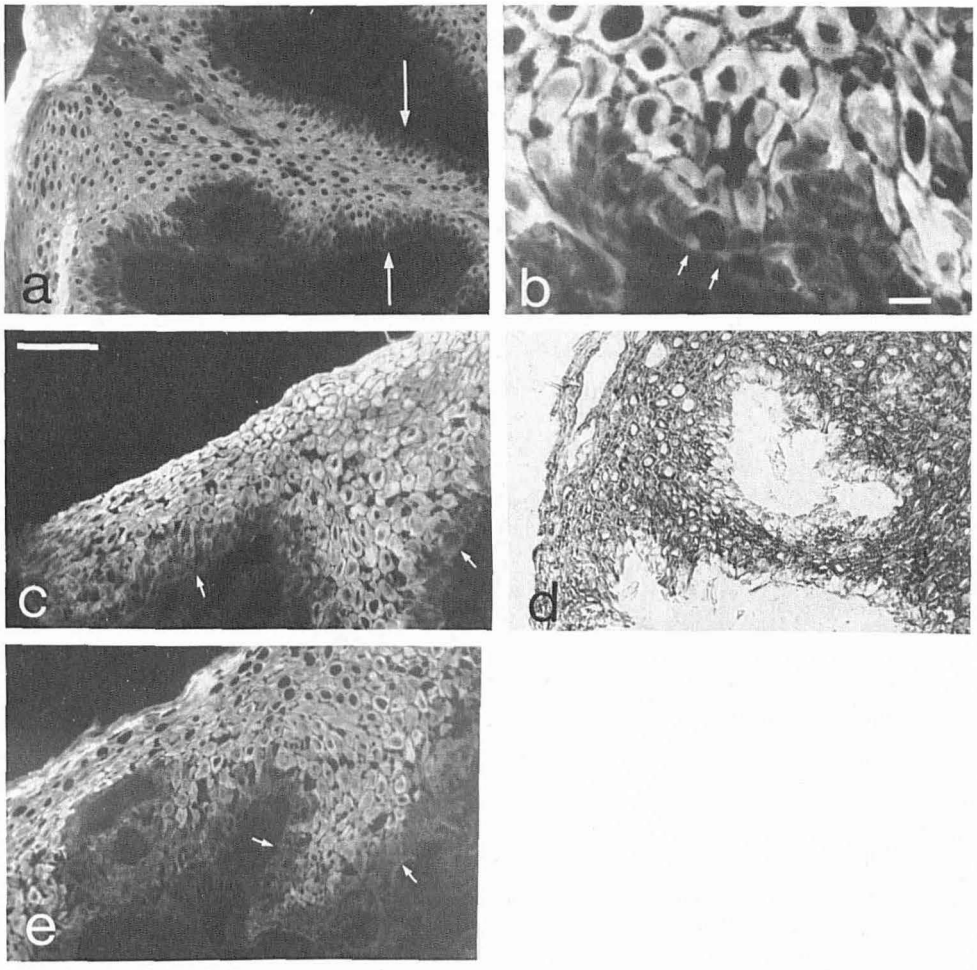

FIG 3. Immunohistochemical staining of verrucae and a seborrheic keratosis by AE1 antibody. *a and b*, Common warts showing suprabasal staining (IF). *c*, Condyloma accuminata also stained suprabasally (IF). *d*, PAP staining of a common wart. *e*, Seborrheic keratosis (IF) looks remarkably similar to (*c*). *Arrows* denote the dermal-epidermal junction. (*a*), (*c*), (*d*), and (*e*) are of the same magnification. *Scale bar* in (*c*) = 50 μm; in (*b*) = 10 μm.

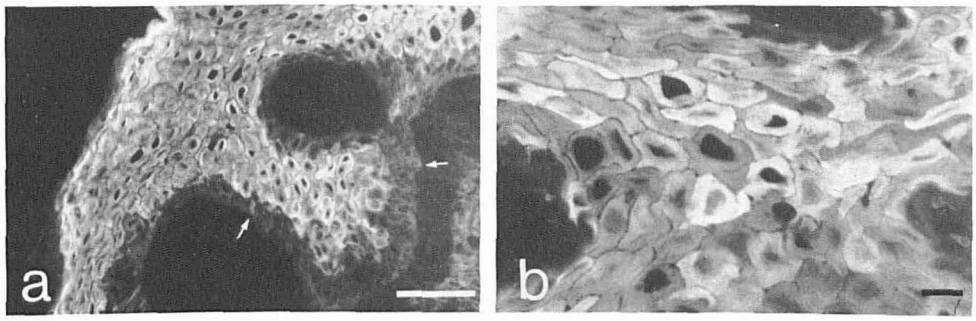

FIG 4. Immunofluorescent staining of an actinic keratosis by AE1 antibody. *a*, Low-power view. Note the suprabasal staining. *Arrows* denote dermal-epidermal junction. *b*, High-power view demonstrating heterogeneity of keratinocyte staining intensity. *Scale bar* in (*a*) = 50 μm; in (*b*) = 10 μm.

2 specimens of BD. The variation in staining intensity among individual cells was much greater than that seen in AK.

Squamous Cell Carcinoma

In 4 SCC, cells with variable staining intensity were present in a highly disorganized fashion (Fig 6), similar to BD. However, in SCC, small clumps of brightly stained cells, which were heterogeneous in size and irregular in shape and thus clearly distinguishable from the cells of normal epidermal appendages, could be seen at the base of the lesion (Fig 6); presumably these represented tumor cells invading into the dermis as seen in neighboring H-E stained sections. Interestingly, the adjacent "uninvolved" epidermis frequently demonstrated the suprabasal staining pattern (see below).

Basal Cell Carcinoma

The 7 BCC that were studied were all of the undifferentiated type. The tumor masses stained weakly with AE1 (Fig 7*a*) and demonstrated a sharp demarcation between the weakly stained tumor nodule and the strongly stained "uninvolved" epidermis (Fig 7*a*,*b*). In addition, similar to SCC, "uninvolved" epidermis directly above and adjacent to the BCC frequently showed suprabasal staining (Fig 7, *large arrows*).

Cultured Human Epidermal Cells as a Model System

Normal human epidermal cells can be grown in culture using lethally irradiated 3T3 cells as a feeder layer [42]. Under these conditions, human epidermal cells can be maintained in a highly proliferative state with a doubling time of 24–30 h [42,

45]. These cells undergo continuous renewal even after reaching confluency, as evidenced by the constant shedding of superficial cornified cells [46–48]. Since many of the diseases that we have studied are known or thought to be hyperproliferative, it was of interest to examine the AE1 staining pattern of cultured human epidermal keratinocytes. An intact sheet of confluent human epidermal cells was released from the dish with Dispase II, embedded in OCT medium, and sectioned [43]. Immunofluorescent staining with AE1 antibody showed the suprabasal staining pattern (Fig 8*a*). In another experiment, a sparse human epidermal culture growing on glass coverslips was fixed with methanol and stained with AE1 antibody (Fig 8*b*). The result indicated that basal cells located at the edge of the growing colonies were either weakly stained or negative, whereas cells above the basal layer were strongly stained, with fluorescently labeled fibers distributed throughout the cytoplasm. These results showed that AE1 antibody produced a suprabasal staining pattern in cultured human epidermal cells similar to that seen in psoriasis and a number of other benign hyperproliferative diseases.

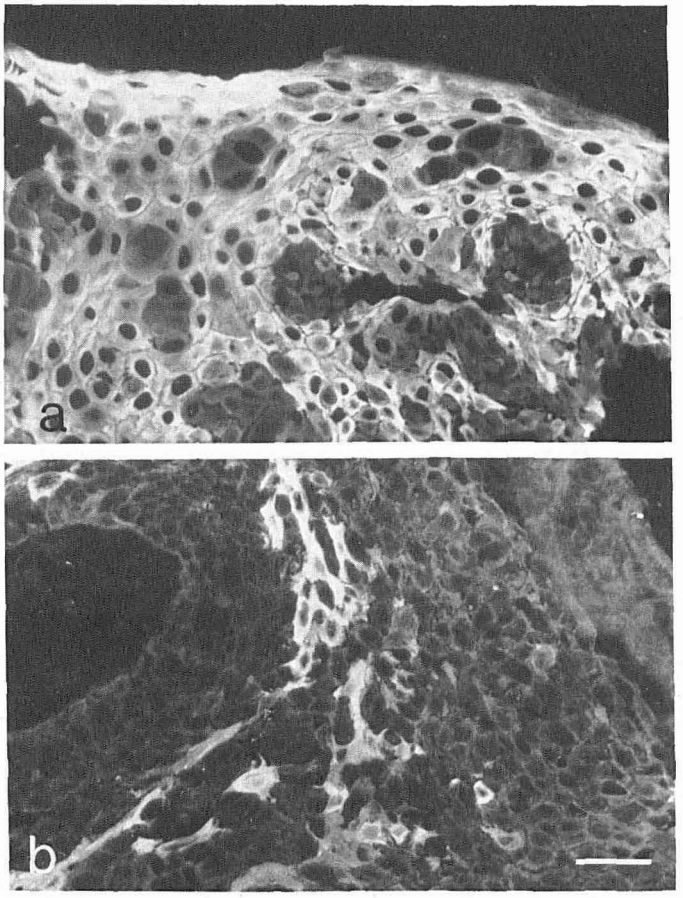

FIG 5. Immunofluorescent staining of Bowen's disease by AE1 antibody. *a*, Markedly heterogeneous staining within the affected epidermis. *b*, A different lesion than (*a*) demonstrating heterogeneous staining. (*a*) and (*b*) are of the same magnification. *Scale bar* = 50 μm.

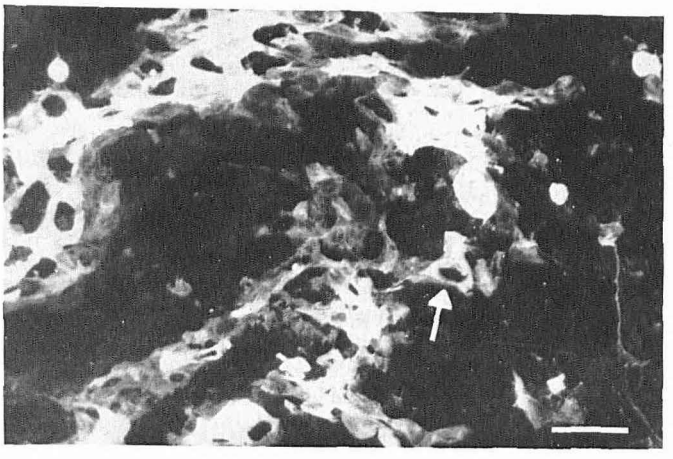

FIG 6. Immunofluorescent staining of a squamous cell carcinoma by AE1 antibody. The picture shows the base of a large tumor with a disorganized staining pattern. The *arrow* indicates an example of the individual keratinocytes detected by AE1 staining. *Scale bar* = 50 μm.

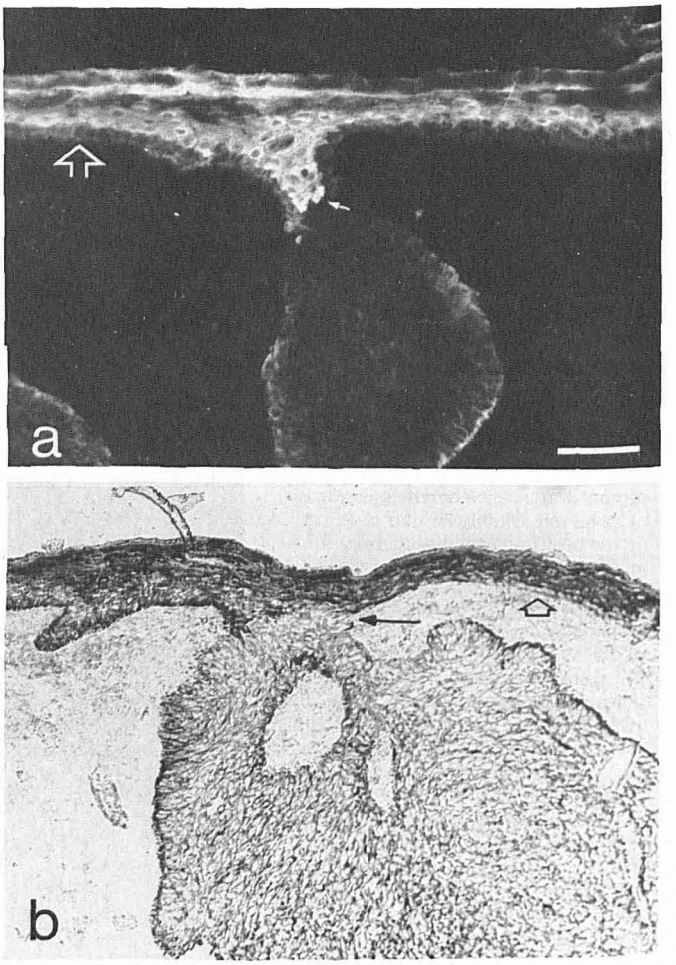

FIG 7. Immunohistochemical staining of a basal cell carcinoma by AE1 antibody. *a*, IF staining. *b*, PAP. These pictures show a tumor mass budding from the undersurface of the epidermis. The *small arrows* indicate the sharp transition between the weakly stained tumor and the intensely stained overlying epidermis. The *larger arrowheads* indicate the suprabasal staining of the overlying, "uninvolved" epidermis; such a "field effect" can be seen in epidermis up to 1 mm from the point of tumor attachment. *Scale bar* = 50 μm.

DISCUSSION

We have used a monoclonal antikeratin antibody (designated AE1) to stain immunohistochemically frozen skin sections from a number of patients with epidermal diseases. While in normal epidermis AE1 antibody selectively stained the basal layer, it produced either "suprabasal" (psoriasis, verrucae, seborrheic keratosis, actinic keratosis), "disorganized" (Bowen's disease, squamous cell carcinoma), or "weak" (basal cell carcinoma) staining patterns in various pathologic conditions.

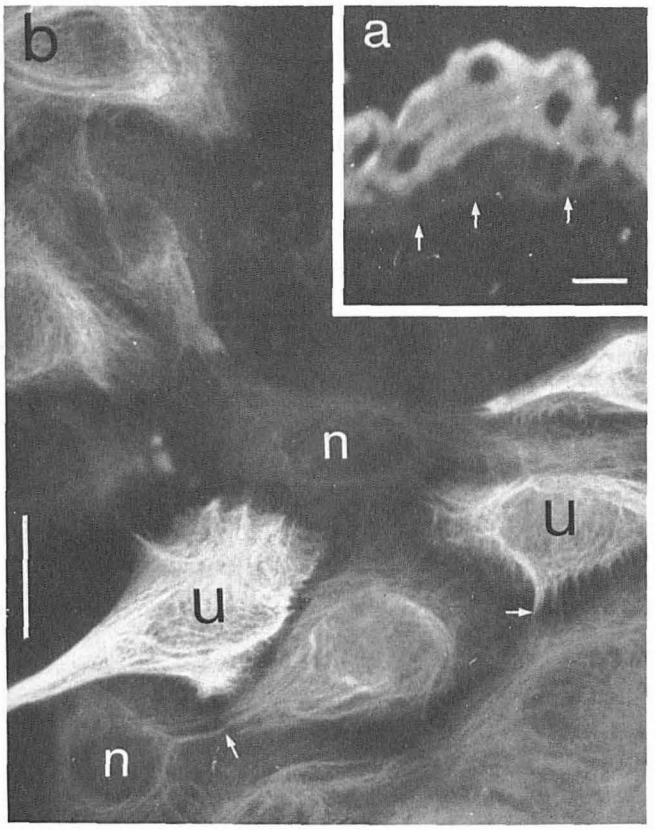

FIG 8. Immunofluorescent staining of cultured normal human epidermal colonies. *a*, Frozen section: An intact sheet of confluent human epidermal cells was released from the Petri dish with Dispase II as described by Green et al [43], embedded in OCT medium, and cryosectioned at 8 μm. A vertical section was stained with AEl antibody. *Arrows* denote the undersurface of the epithelium originally in contact with the Petri dish. Note the suprabasal staining pattern. *Scale bar* = 25 μm. *b*, Coverslip: A sparse, exponentially growing culture of human newborn foreskin epidermal cells grown on glass coverslip was fixed with methanol and stained with AEl. Note that basal cells (nuclei labeled *n*) were either weakly stained or negative, while upper cells (labeled *u*) showed strongly stained keratin fibers (*arrows* indicate desmosomal type cell-cell junctions). *Scale bar* = 25 μm.

The most striking and frequently observed pattern was the suprabasal one, which appeared common to many pathologic processes including psoriasis, warts, SK, AK, as well as the epidermis in close proximity to some epidermal neoplasms. The mechanism for such an abnormal suprabasal staining pattern in diseases has not yet been established and is currently under investigation. Possible mechanisms include the synthesis of new keratin species, the modification of keratins leading to the exposure or unmasking of additional AEl antigenic site(s) in cells above the basal layer, as well as the loss or masking of AEl antigens in the basal layer [6]. Whatever the mechanism might be, it is probably related to a hyperproliferative state of the keratinocytes and cannot be disease-specific, since the same suprabasal staining pattern was observed in a wide variety of epidermal abnormalities with diverse etiology and pathogenesis, as well as in cultured human epidermal colonies.

We found that the epidermis adjacent to neoplasms including BCC and SCC exhibited abnormal suprabasal AEl staining. This observation is consistent with an earlier report by Wolf and Bystryn who demonstrated an altered expression of some unknown antigens, as defined by autoimmune antisera, in the epidermis adjacent to BCC [49]. Although the mechanism of such a "field effect" is not yet known, we believe that this phenomenon is a nonspecific response of the epidermis to a proliferative or inflammatory stimulus.

A "disorganized" staining pattern was observed in some malignant diseases including BD and SCC. In these diseases the intensity of AEl staining was highly variable and the suprabasal pattern was essentially lost. These results suggest that in certain epidermal malignancies there may be additional alterations or perturbations in the program of keratin expression.

In SCC, groups of strongly stained cells, presumably representing tumor cells, could be easily identified by AEl in the reticular dermis (Fig 6). These results are consistent with earlier findings using conventional antikeratin antisera [32,50–53] (also J. Robinson, Northwestern University, personal communication) and suggest that antikeratin staining can be useful for detecting carcinoma cells invading into the surrounding (keratin-negative) mesenchymal tissues. However, in such an application, a mixture of several monoclonal antikeratin antibodies or a conventional antiserum known to react with all epidermal cells or SCC cells should be used to assure the detection of all tumor cells.

In summary, we showed that the AEl staining pattern varies depending on the disease state of the epidermis. The suprabasal staining pattern was the most frequently encountered and was observed in many hyperproliferative epidermal disorders as well as in cultured epidermal colonies. These observations suggest a common alteration in keratin expression, possibly related to a hyperproliferative state of the keratinocyte. Experiments are underway to further investigate this possibility.

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