Immunolocalization of Keratin Polypeptides in Human Epidermis Using Monoclonal Antibodies

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ABSTRACT Three monoclonal antibodies (AE1, AE2, and AE3) were prepared against human epidermal keratins and used to study keratin expression during normal epidermal differentiation. Immunofluorescence staining data suggested that the antibodies were specific for keratin-type intermediate filaments. The reactivity of these antibodies to individual human epidermal keratin polypeptides (65-67, 58, 56, and 50 kdaltons) was determined by the immunoblot technique. AE1 reacted with 56 and 50 kdalton keratins, AE2 with 65-67 and 56-kdalton keratins, and AE3 with 65-67 and 58 kdalton keratins. Thus all major epidermal keratins were recognized by at least one of the monoclonal antibodies. Moreover, common antigenic determinants were present in subsets of epidermal keratins.

To correlate the expression of specific keratins with different stages of in vivo epidermal differentiation, the antibodies were used for immunohistochemical staining of frozen skin sections. AE1 reacted with epidermal basal cells, AE2 with cells above the basal layer, and AE3 with the entire epidermis. The observation that AE1 and AE2 antibodies (which recognized a common 56 kdalton keratin) stained mutually exclusive parts of the epidermis suggested that certain keratin antigens must be masked *in situ*. This was shown to be the case by direct analysis of keratins extracted from serial, horizontal skin sections using the immunoblot technique.

The results from these immunohistochemical and biochemical approaches suggested that: (*a*) the 65- to 67-kdalton keratins were present only in cells above the basal layer, (*b*) the 58-kdalton keratin was detected throughout the entire epidermis including the basal layer, (*c*) the 56-kdalton keratin was absent in the basal layer and first appeared probably in the upper spinous layer, and (*d*) the 50-kdalton keratin was the only other major keratin detected in the basal layer and was normally eliminated during s. corneum formation. The 56 and 65-67-kdalton keratins, which are characteristic of epidermal cells undergoing terminal differentiation, may be regarded as molecular markers for keratinization.

Keratins are a family of water-insoluble proteins of 40,000 to 70,000 daltons (40 to 70 kdaltons [kd]). These proteins form tonofilaments (a class of intermediate filament) in epidermis (2, 4, 6, 9, 13, 19, 20, 21, 27, 37, 39, 41, 46, 49, 54) as well as in almost all other epithelia (10–12, 30, 36, 45, 47, 48).

The process of normal epidermal differentiation (keratinization) is characterized by a series of morphological and biochemical changes as cells progress from the germinative basal layer through the spinous and granular layers to the outer cornified layer (55). Analysis of keratins extracted from horizontal sections of the epidermis suggests that cells of inner layers contain primarily small keratins, whereas cells of outer layers contain large keratins in addition to small ones (14, 40). Furthermore, antisera specific for the high molecular weight (65–67 kd) keratins demonstrate preferential binding to cells above the basal layer (44; cf. 52, 53). These data suggest that keratin composition changes during epidermal differentiation and, more specifically, that the 65- to 67-kd keratins first appear in the spinous layer. Other keratins of the epidermis, however, have not been localized precisely and thus the functional significance of these keratins remains unclear.

To facilitate the immunolocalization of specific keratins in

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epidermis, we have prepared monoclonal antibodies to human epidermal keratins using the hybridoma technique (23). In this paper we describe the properties of three of these antibodies and their use for studying the expression of keratin antigens during epidermal differentiation. Using a combination of biochemical and immunological techniques, we demonstrated that a 50 and a 58-kd keratin were present in all living layers including the relatively undifferentiated basal cell layer, whereas a 56-kd keratin and 65- to 67-kd keratins were associated only with more differentiated cells above the basal layer. The latter keratins may therefore be regarded as molecular markers of keratinization.

MATERIALS AND METHODS

Keratin Antigens

Total keratin was isolated from human epidermal callus as described previously (46). 1.3 mg of such keratins in 0.1 ml of 8 M urea, 25 mM Tris-HCl (pH 7.4) was mixed with SDS and 2-mercaptoethanol (final concentrations 7.5 and 1%, respectively). After heating to 65°C for 10 min, the denatured keratins were dialyzed against distilled water and used as the antigen.

Hybridoma Production

An aliquot of SDS-denatured total callus keratin (40 μg) was diluted to 0.2 ml with phosphate-buffered saline (PBS), mixed with 0.2 ml of Freund's complete adjuvant, and injected subcutaneously at multiple sites into each of ten female BALB/c mice. Four weeks later, the same procedure was repeated except that Freund's incomplete adjuvant was used. After an additional 3 wk, 40 μg of keratin in PBS was injected intravenously. The mice were sacrificed 3 d later and the spleen cells were isolated for hybridization.

P3 × 63 Ag8 myeloma cells (P3 cells; kindly provided by Dr. C. Milstein) and mouse spleen cells (2×10^7 and 2×10^8 cells, respectively) were fused in the presence of 50% polyethylene glycol 1500 (Aldrich Chemical Co., Milwaukee, WI) for 1 min at 37°C. After washing with PBS, cells were distributed into 96 wells (24 wells per plate, Costar, Data Packaging, Cambridge, MA) in Dulbecco's modified Eagle's medium containing 10^{-4} M hypoxanthine, 10^{-6} M aminopterin, and 1.6×10^{-5} M thymidine (HAT medium; 28) in the presence of lethally irradiated (5,000 Rads) 3T3 fibroblasts (plated at 1.5×10^4 cells/cm^2).

The initial cloning of hybridoma cells was done in soft agar (22) using 3T3 feeders. The second and third clonings were done by plating an average of one cell per three wells, without soft agar, again with 3T3 feeders.

Culture medium conditioned by repeatedly cloned hybridoma cells was used as the source of the monoclonal antibody.

Assays for Antikeratin Antibody Activity

Antikeratin antibody activity was assayed by an enzyme-linked immunosorbent assay (ELISA). SDS-denatured callus keratin (see above) was diluted with 60 mM $NaHCO_3$ (pH 9.6) to a final concentration of 6.5 μg/ml and used as antigen. Each well of the ELISA plate (96 wells/plate, Dynatech Corp., Alexandria, VA) was sequentially treated with 0.1 ml of the following: (*a*) keratin antigen (4°C, overnight); (*b*) 0.5% bovine serum albumin, 100 mM sodium phosphate buffer (pH 7.2), and 0.05% Tween 20 (buffer A; 25°C, 1 h) to saturate the nonspecific protein binding sites; (*c*) hybridoma culture medium (37°C, 1 h); (*d*) sodium phosphate buffer (25°C, three times for 10 min each); (*e*) peroxidase-conjugated goat anti-mouse IgG (ENZabody, Litton Bionetics, Kensington, MD; diluted 1:100 in buffer A; 37°C, 1 h); and (*f*) a substrate solution which was freshly prepared by dissolving 80 mg of 5-aminosalicylic acid (Sigma Chemical Co., St. Louis, MO) in 100 ml of warm (75°C) distilled water, adjusting to pH 6.0 at room temperature with 1 M NaOH, and then adding 0.01 ml of 0.05% H_2O_2.

Wells containing culture medium with anti-keratin activity developed a dark brown color within 5–10 min. The reaction was terminated by adding 0.025 ml of 1 M NaOH per well. This assay was at least 200 times more sensitive in detecting antikeratin activity than the indirect immunofluorescence staining assay using cultured human epidermal cells as a substrate; a conventional mouse antikeratin antiserum with a titer of ~1:50 by immunofluorescence gave a specific, positive reaction by ELISA at a dilution of 1:10,000.

Extraction of Keratin From Human Epidermis

Human abdominal skin was obtained from autopsy, frozen on dry ice, and used immediately. The epidermis was scraped from the frozen tissue, minced, and homogenized in a solution of 25 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), antipain (10 μg/ml; Sigma Chemical Co.), and pepstatin (5 μg/ml; Sigma Chemical Co.). After centrifugation (10,000 *g* for 10 min at 4°C), the supernatant was discarded and the pellet was further extracted as follows: (*a*) Samples to be analyzed by SDS PAGE were extracted (95°C, 5 min) with 1% SDS, 25 mM Tris-HCl (pH 7.4) to obtain keratins of the living layers (1, 40, 46; see Results). After centrifugation, the pellets were further extracted with 1% SDS and 5% 2-mercaptoethanol in 25 mM Tris-HCl (pH 7.4) to obtain keratins of the stratum corneum (s. corneum). (*b*) Samples to be analyzed on two-dimensional gels (31, 46) were similarly extracted except that a fresh solution of 8 M urea replaced SDS and the extraction was carried out at room temperature.

Immunofluorescence Staining

Human epidermal cells from newborn foreskin, grown in the presence of 3T3 feeder cells (35), were fixed and stained by indirect immunofluorescence (47). Frozen tissue sections (6–8 μm) were stained by indirect immunofluorescence (47, 48) or by the peroxidase-anti-peroxidase (PAP) technique (43).

In experiments designed to "unmask" the keratin antigens, frozen skin sections were air-dried with a fan for 30 min, and preincubated for 70 min at 37°C with the following reagents: 100 mM Tris-HCl (pH 7.5); 0.0001 or 0.005% chymotrypsin (in 100 mM Tris-HCl, pH 7.5); or 0.0001 or 0.005% trypsin (in 100 mM Tris-HCl, pH 8.0); 0.0001% heparinase (in 100 mM Na acetate, pH 6.5); 0.0001% bacterial alkaline phosphatase (in 100 mM Tris-HCl, pH 8.0); 0.0001% potato acid phosphatase (in 100 mM Na acetate, pH 5.0); 1% Triton X-100 (in 100 mM Tris-HCl pH 7.5); and 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 2 mM PMSF, 10 μg/ml antipain, and 5 μg/ml pepstatin (in 100 mM Tris-HCl, pH 7.5). After preincubation, slides were treated with 4% normal goat serum and stained with antibodies as described above.

Binding of Antibodies to Antigens by Immunoblot Technique

Proteins from unstained polyacrylamide gels were transferred electrophoretically (50) onto nitrocellulose paper (Millipore Corp., Bedford, MA) using an E-C blotting apparatus (2.5 h at 4°C with a power supply setting of 65%). To visualize protein bands, the blot was stained with Fast green (0.1% in 50% methanol/10% acetic acid) for 3–10 min, destained in 50% methanol/10% acetic acid for 5–10 min, and rinsed with PBS. Fast green staining afforded rapid visualization of protein bands without interfering with subsequent antibody/PAP staining. The blot was then incubated sequentially in 3% BSA for 1 h at 37°C to saturate the protein binding sites, in 4% goat serum for 30 min at 37°C, and in hybridoma culture medium containing monoclonal antibody for 1 h. After washing with PBS, the blot was treated with goat anti-mouse IgG (Miles Laboratories, Inc., Elkhardt, IN; 1:20 diluted with 3% BSA) for 30 min at 37°C, washed with PBS, and incubated with mouse PAP (Sternberger-Meyer Co., Jarretsville, MD; 1:100 diluted) for 60 min at 25°C. The blot was washed with 50 mM Tris-HCl (pH 7.6) at 25°C for 15 min, and incubated with a freshly prepared substrate solution containing 50 mM Tris-HCl (pH 7.6), 3,3′-diaminobenzidine-HCl (0.05 mg/ml), and 0.01% H_2O_2 at room temperature for 3–20 min. Finally, the blot was rinsed with water and air-dried.

PAP staining with monoclonal antibodies was highly sensitive in detecting the presence of antigen bands (18, 50). For optimal resolution, we typically applied 20 to 60% less protein onto gels to be analyzed by immunoblotting than gels to be directly stained with Coomassie Blue.

Gel Electrophoresis

One-dimensional SDS PAGE was performed according to Laemmli (24, 46). Two-dimensional gel electrophoresis was done by the method of O'Farrell (31, 46).

Labeling of Cell Proteins

Cells were incubated in a medium containing [^{35}S]methionine (sp act, 1 μCi/μg; 31 μCi/ml medium) for 4 h at 37°C (46).

RESULTS

Preparation of Monoclonal Antibodies

Hybridoma cells were produced by fusing P3 myeloma cells with spleen cells from BALB/c mice immunized with SDS-denatured human epidermal keratins. In two independent fu-

sion experiments, growth of hybridoma cells was observed in about 160 out of 200 wells. Cells from six wells were found to produce antikeratin activity when assayed by ELISA (see Materials and Methods). After repeated cloning, three anti-keratin-producing hybridoma lines, designated AE1, AE2, and AE3, were isolated and their antibodies characterized.

The monoclonal nature of the hybridoma cell lines was established by three criteria. First, each cell line was cloned at least three times. Second, one- and two-dimensional gel electrophoresis of [^{35}S]methionine-labeled proteins secreted by each cell line demonstrated only one additional immunoglobulin light chain and one extra heavy chain when compared with proteins produced by parent P3 myeloma cells. The additional chains made by the three hybridoma lines were slightly different in size, but sister clones derived from a given line produced identical patterns (not shown). Finally, antibodies produced by sister clones yielded identical results when tested with extracted keratins and with frozen skin sections.

The specificity of the antibodies was determined by immunofluorescence staining of various types of cultured cells and of frozen skin sections. In cultured human epidermal cells, all three antibodies decorated a network of cytoplasmic fibers (Fig. 1 *a–c*) anchored at desmosomal cell-cell junctions (Fig. 1, arrows). Culture medium conditioned by P3 myeloma cells produced no such staining (Fig. 1 *d*). Furthermore, the fibrous staining pattern produced by the antibodies was not altered significantly by pretreatment of the cells with cytochalasin B (10 μg/ml, 1 h) or colcemid (10 μg/ml, 4 h), indicating that the major staining reaction was not due to microfilaments or microtubules (32). None of the three antibodies produced significant staining in nonepithelial cells, including 3T3 mouse fibroblasts, human embryonic lung fibroblasts (WI-38), human skin fibroblasts, human neuroblastoma cells (IMR-32), and human myeloma cells (RPMI 8226). In frozen sections of human skin, each antibody stained only the epidermis (see below), with no detectable staining of any dermal components including fibroblasts, endothelial cells, muscle cells, blood cells, or nerves. These results were similar to those obtained with conventional antikeratin antisera (11, 12, 17, 36, 47, 48), and suggested that the monoclonal antibodies were specific for keratin-type intermediate filaments.

Binding of Monoclonal Antibodies to Individual Keratins Isolated from Living Layers of the Epidermis

To determine the specificity of the monoclonal antibodies to individual keratin polypeptides, we prepared keratins from human epidermis and analyzed them using the immunoblot technique (50). Since keratins are known to undergo significant modifications during s. corneum formation (14, 40), keratins from living layers and s. corneum were isolated and examined separately.

Keratins of the living layers are not cross-linked by inter-

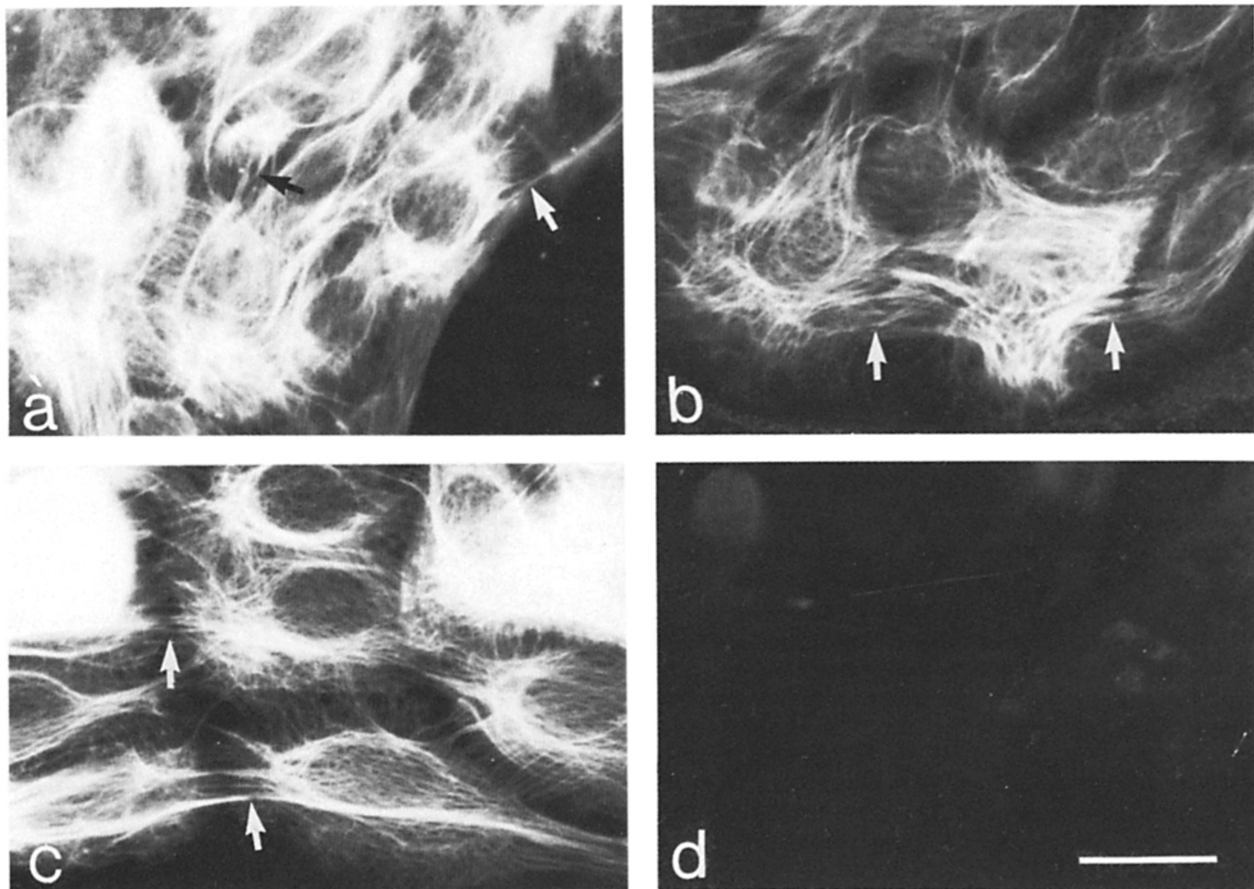

FIGURE 1 Indirect immunofluorescence staining of cultured human epidermal cells. Tertiary cultures of human epidermal cells derived from newborn foreskin were grown on glass cover slips, fixed with methanol and stained with mouse antibodies by indirect immunofluorescence. (*a*) AE1 antibody. (*b*) AE2 antibody. (*c*) AE3 antibody. (*d*) P3 control. Arrows indicate cell-cell junctions presumably containing desmosomes. All pictures are of the same magnification. Bar, 25 μm. × 760.

molecular disulfide bonds (1, 40, 45) and therefore can be selectively extracted with 1% SDS in the absence of a reducing agent. When such keratins were analyzed by SDS PAGE, four major components of 50, 56, 58, and 65–67 kdaltons were observed (see Fig. 2, lane *1*).

Immunoblot analysis (see Materials and Methods) showed that AE1 antibody reacted predominantly with the 50 and 56-kd keratins; occasionally, a weak 48-kd band was also detected (Fig. 2; lane *4*; also see Fig. 3). AE2 stained high molecular weight (65–67 kd) keratins and the same 56-kd band recognized by AE1 (Fig. 2, lane *7*). AE3 reacted with the same 65- to 67-kd keratins recognized by AE2 and, in addition, the 58-kd keratin (Fig. 2; lane *10*). None of the antibodies showed significant binding to any water-insoluble proteins of WI-38 human fibroblasts, including actin or vimentin, an intermediate filament protein characteristic of mesenchymal cells (see Fig. 2, lanes *3*, *6*, *9*, and *12*; and reference 11). Control experiments with P3-conditioned medium demonstrated no staining of any protein bands from epidermal cells or fibroblasts (not shown).

To further characterize the antigens recognized by these antibodies, keratins were separated by two-dimensional gel electrophoresis (Fig. 3 *a*). After Fast green staining, the 50 and variable 48-kd components appeared as two closely associated spots which were slightly more acidic than actin. The 56-kd keratin formed a major spot isoelectric with the 50-kd keratin. No well-defined spots corresponding to the 58-kd keratin or 65–67 kd keratins were observed, suggesting that the pI's of these keratins may be outside the pH range of the gels (46).

Keratins separated by two-dimensional gel electrophoresis were reacted with the monoclonal antibodies by the immunoblot technique. Such experiments demonstrated that the 50 and 48-kd spots were recognized by AE1 (Fig. 3 *b*), whereas the 56-kd keratin spot was stained by both AE1 and AE2 (Fig. 3 *b* and *c*). Although numerous minor spots of 58–67 kd were detected by AE3 (Fig. 3 *d*) and weakly by AEZ (Fig. 3 *c*), these spots did not correspond to any major keratins detected by fast green staining (Fig. 3 *a*).

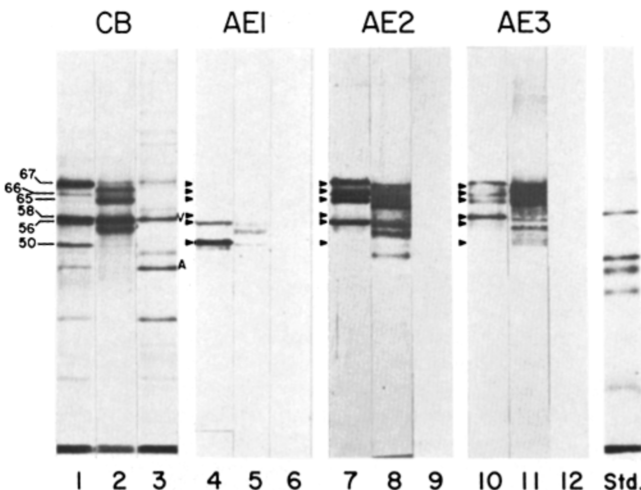

FIGURE 2 Binding of monoclonal antibodies to human epidermal keratins separated by SDS gel electrophoresis. Water-insoluble cytoskeletal proteins from the living layers of human abdominal epidermis (lanes *1*, *4*, *7*, and *10*), the cornified layers (lanes *2*, *5*, *8*, and *11*), and cultured human fibroblasts (WI-38; lanes *3*, *6*, *9*, and *12*) were separated on 12.5% polyacrylamide gels. Lanes *1–3* were stained directly with Coomassie Blue. Samples on lanes *4–12* were electrophoretically transferred onto nitrocellulose paper (48) and stained by PAP technique with AE1 (lanes *4–6*), AE2 (lanes *7–9*) and AE3 (lanes *10–12*). (*Std.*) molecular weight standards: bovine serum albumin, catalase, fumarase, ovalbumin, aldolase, chymotrypsinogen A, and B-lactoglobulin. *V* and *A* of lane *3* represent vimentin and actin, respectively. Molecular weight values at left are × 10^{-3}.

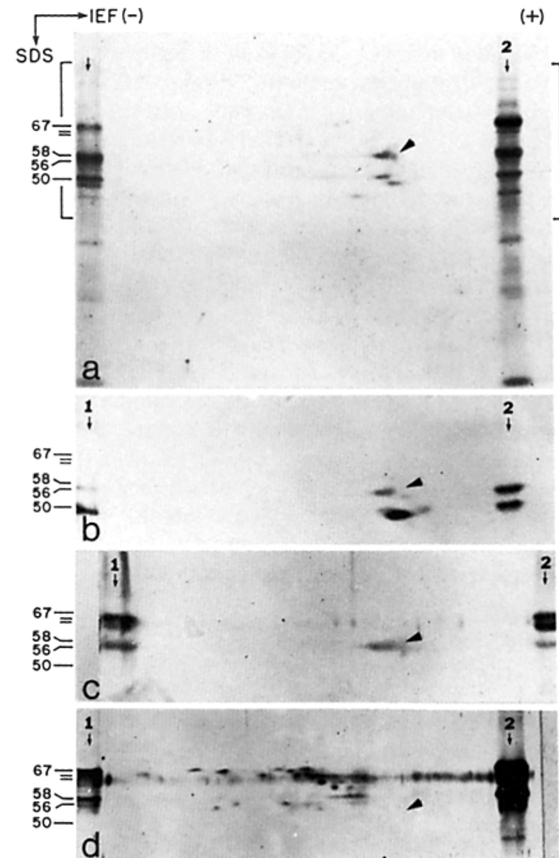

FIGURE 3 Binding of monoclonal antibodies to keratins separated by two-dimensional PAGE. Water-insoluble proteins were extracted from human abdominal epidermis with 8 M urea, separated first by isoelectric focusing (IEF) and then by SDS-gel electrophoresis (31, 46). The proteins were transferred from the gel to nitrocellulose paper and stained with (*a*) Fast green, (*b*) AE1 (*c*) AE2, or (*d*) AE3. Only the bracketed area in *a* is shown in *b*, *c*, and *d*. Lanes on the sides of each gel, as denoted by arrows *1* and *2*, are samples of human abdominal epidermis extracted with 8 M urea or 1% SDS, respectively, separated only by the second dimensional SDS-gel electrophoresis. The position of the 56,000-mol wt keratin is shown by an arrowhead in each immunoblot for orientation. Molecular equivalents at left are × 10^{-3}.

These results showed that the three monoclonal antibodies recognized different but partially overlapping subsets of epidermal keratins. In addition, the data indicated that common antigenic determinants must be present in multiple keratin components (13, 20, 27, 34, 41, 49), and that most, if not all, major epidermal keratins were recognized by at least one of the three monoclonal antibodies.

Binding of Monoclonal Antibodies to S. Corneum Keratins

To investigate changes in keratins during s. corneum formation, we isolated the disulfide-cross-linked keratins of cornified cells (1, 45) and characterized them using the monoclonal antibodies. Coomassie Blue-stained gels of such preparations showed that the 50-kd keratin was greatly diminished (Fig. 2,

lane 2; references 14 and 38); most other keratins appeared slightly smaller than the corresponding bands of the living layers (1, 14, 38). When s. corneum keratins were analyzed by immunoblot technique using AE1, only small amounts of the 50 and 56-kd keratins of the living layers were detected (Fig. 2, lane *5*). Instead, the antibody recognized a major component of 55-kd. This result was in agreement with earlier data (14) and provided direct evidence that the 55-kd keratin of s. corneum was antigenically related to the 56-kd keratin of the living layers. AE2 antibody stained the high molecular weight bands. This antibody also reacted with a 56-kd keratin as well as the 55-kd band recognized by AE1 (Fig. 2, lane *8*), thus confirming the relatedness of the 55 and 56-kd keratins. Finally, both AE2 and AE3 detected in s. corneum samples some minor, small molecular weight bands (46–53 kd) which presumably represent keratin fragments.

These results confirmed and extended earlier observations by Fuchs and Green (14), and suggested that the 50-kd keratin was virtually absent in s. corneum and that a 55-kd s. corneum keratin was derived from the 56-kd keratin.

Immunohistochemical Staining of Human Epidermis

To correlate the expression of specific keratins with different stages of epidermal differentiation, we stained frozen sections of human skin with each monoclonal antibody by indirect immunofluorescence or the PAP technique.

AE1 Antibody

By the PAP technique, AE1 stained predominantly epidermal basal cells in skin from forearm (Fig. 4 *b*), newborn foreskin (Fig. 4 *c*), abdomen, leg, knee, and back (not shown). No staining was detectable in cells above the basal layer, nor in any dermal components. Immunofluorescence microscopy of newborn foreskin confirmed that the antibody reacted predominantly with epidermal basal cells. At higher magnification, such staining appeared cytoplasmic and fibrous in nature (Fig. 4 *e*). These results contrasted sharply with those obtained with conventional mouse antitotal-keratin antisera which either stained the entire epidermis, or preferentially stained cells above the basal layer (Fig. 4 *d*). Control experiments using normal mouse serum or medium conditioned by P3 myeloma cells produced no detectable staining in similar skin sections (Fig. 4 *a*). Since AE1 was specific for 50 and 56-kd keratins (Fig. 2, lane *4*), such results showed that at least one of these two keratins must exist in the epidermal basal layer.

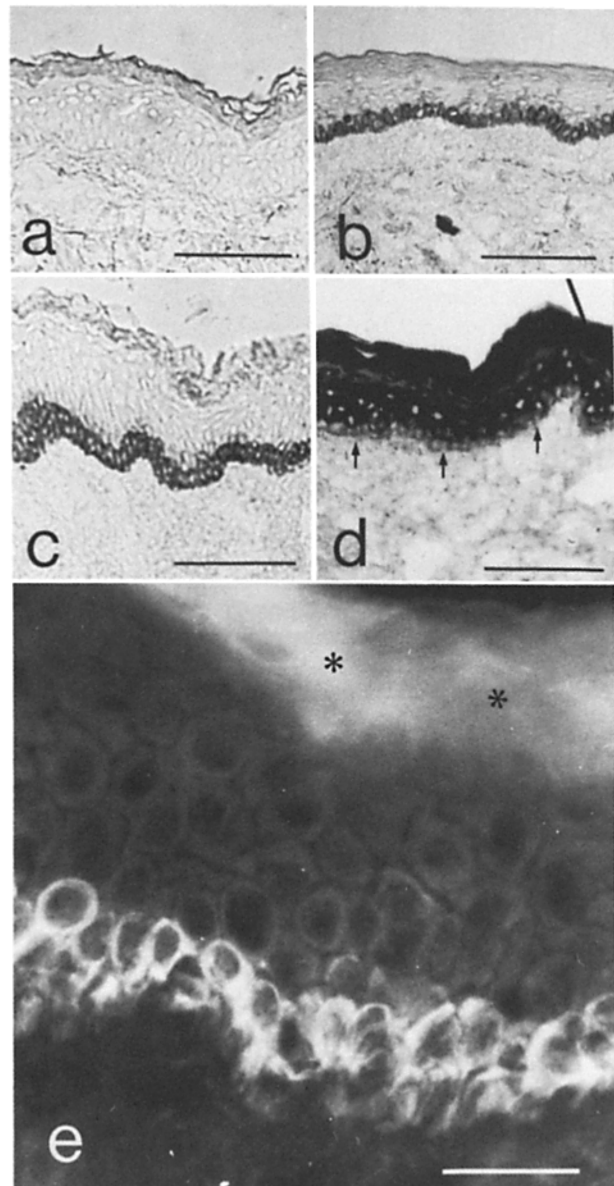

FIGURE 4 Immunohistochemical staining of sections of normal human skin with AE1. Frozen sections of human skin were stained by PAP technique (*a–d*) or by indirect immunofluorescence (*e*) with various antibodies. (*a*) Forearm, P3 control. (*b*) Forearm, AE1. (*c*) Exterior portion of newborn foreskin, AE1. Note the strong staining of basal cells in *b* and *c*. (*d*) Foreskin (exterior portion), a mouse antiserum to total human callus keratins (1/100 diluted with PBS). Arrows indicate the epidermal-dermal junction. Note the preferential staining of epidermal cells above the basal layer. (*e*) Newborn foreskin (exterior portion), AE1. Note the staining of fibrous structures in the cytoplasm of basal cells. Asterisks denote the nonspecific staining of s. corneum (10, 47). It should be noted that, by the less sensitive immunofluorescence technique, only weak staining of epidermal basal cells was observed when adult skin was used. Bars, (*a–d*) 100 μm. Bar, (*e*) 25 μm.

AE2 Antibody

AE2 reacted preferentially with epidermal cells above the basal layer (Fig. 5 *a*). The boundary between the unstained basal cells and the strongly stained suprabasal cells was usually quite sharp. Occasionally, however, a few negative cells were observed immediately above the basal layer (Fig. 5 *b*, arrows); presumably these represented newly differentiated cells which did not yet express the AE2 keratin antigens. These results were similar to those produced by guinea pig antisera specific for the 65- to 67-kd keratins (44, 52, 53).

AE3 Antibody

AE3 reacted with the entire epidermis (Fig. 6 *a*). Occasionally suprabasal cells stained slightly stronger than basal cells (Fig. 6 *b*). Since this antibody was specific for the 65- to 67-kd keratin triplet and a 58-kd keratin, and since the 65- to 67-kd keratin has been localized to suprabasal cells (44, 52, 53; cf. 14), this result suggested that the 58-kd keratin was present in basal cells (for additional data, see below).

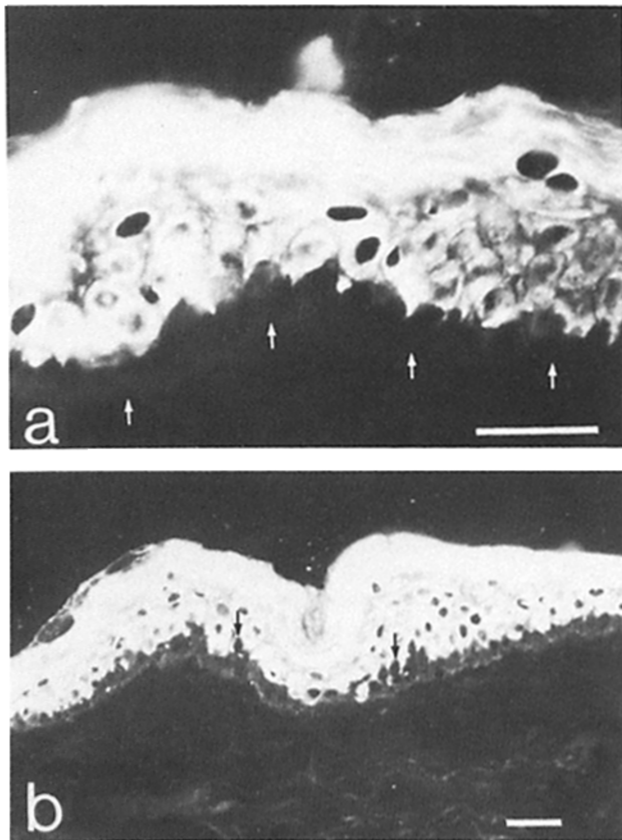

FIGURE 5 Immunofluorescence staining of frozen sections of human abdominal skin with AE2 antibody. (*a*) High magnification. Arrows indicate the epidermaldermal junction. Note the intense staining of suprabasal cells. (*b*) Low magnification. Arrows mark the few negative cells situated above the basal layer. Bars, 25 μm.

Immunoblot Analysis of Keratins Isolated from Horizontal Sections of Epidermis

Immunohistochemical staining of frozen skin sections with individual monoclonal antibodies suggested that the 58-kd keratin, and the 50 and/or 56-kd keratins, were localized in basal cells. The limitation of this approach was demonstrated, however, by the observation that AE1 and AE2 antibodies, both of which recognized the same 56-kd keratin on SDS gels, stained mutually exclusive parts of the epidermis (basal vs. suprabasal). This result could be due to masking of some keratin antigens in tissue sections (10, 16, 41). To test this possibility, frozen skin sections were treated with a variety of proteases, glycosidases, or phosphatases before antibody staining (see Materials and Methods). No pretreatment, however, altered the antibody staining patterns.

As an alternative approach to resolve the keratins in different epidermal layers, we prepared horizontal sections of human heel epidermis (14). Keratins were extracted from serial sections, separated by SDS PAGE and transferred to nitrocellulose paper. Fast green staining of the blot revealed minor but reproducible changes in keratin patterns from different sections of the epidermis (Fig. 7 *a*).

Subsequent staining of the same blot with AE1 antibody revealed clear cut changes in certain keratins during epidermal differentiation. A prominent 50-kd keratin (and a strong 48-kd keratin in heel) were detected by AE1 in sections containing all epidermal layers, including the innermost region (Fig. 7 *b*, lanes *1* and *2*). In contrast, the 56-kd keratin was detected by AE1 (and AE2, not shown) only in sections containing upper epidermis (lanes *3*, *4*, and up). In fractions containing s. corneum (lane *8* and up), a 55-kd keratin was also recognized by AE1 and AE2. This result again suggested that the 55-kd keratin was derived from the 56-kd band during s. corneum formation (Fig. 7 *b*, lanes *8–13*; also see Fig. 2, lanes *5* and *8*).

When the same immunoblot was subsequently stained with AE3 (Fig. 7 *c*), the 58-kd keratin was readily detected in innermost epidermal fractions enriched with basal cells (Fig. 7 *c*, lanes *1* and *2*). Additional experiments showed that this keratin was present throughout the epidermis. The 65- to 67-kd keratins were weakly stained in section 1 and strongly stained in all other sections.

The thickness of heel epidermis provided maximum resolution of various cell layers, and the scarcity of hair follicles permitted straightforward interpretation of the data. Keratins extracted from horizontal sections of human abdominal epidermis produced similar results, with two exceptions: abdominal epidermis demonstrated no significant amount of the 48-kd keratin so prominent in heel, and showed virtual elimination of the 50-kd band in s. corneum (Fig. 2).

DISCUSSION

Expression of Keratin Antigens during Epidermal Differentiation

We have prepared and characterized three monoclonal anti-

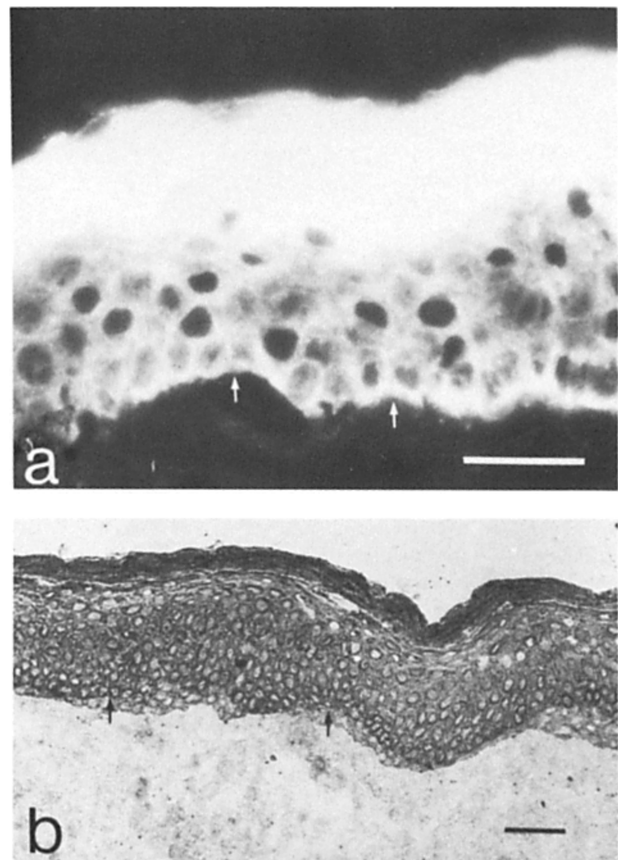

FIGURE 6 Immunohistochemical staining of sections of human abdominal skin with AE3 antibody. (*a*) Indirect immunofluorescence staining. Arrows indicate the epidermal-dermal junction. Note the staining of the entire epidermis. (*b*) Peroxidase-antiperoxidase staining. Arrows indicate the demarcation between the basal and suprabasal compartments of the epidermis. Note that some suprabasal cells stained slightly stronger than the basal cells. Bar, 25 μm.

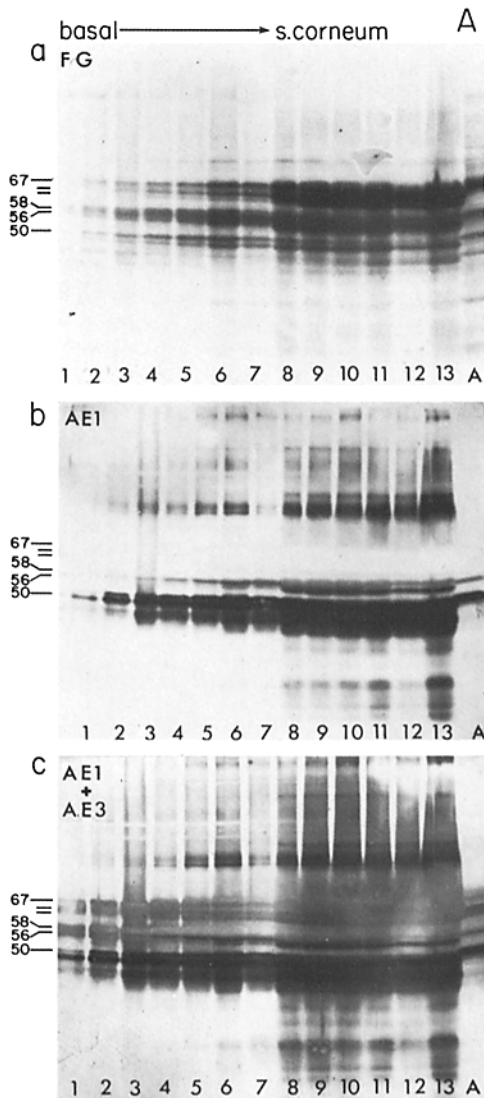

FIGURE 7 Immunoblot analysis of keratins in serial, horizontal sections of human heel epidermis. A specimen of heel skin (3 mm in diameter, obtained from autopsy) was frozen in liquid N_2, embedded epidermal side up in OCT medium, and sectioned (20-μm thick) parallel to skin surface (14). Each section was first extracted with 25 mM Tris-HCl (pH 7.4) containing several protease inhibitors (see Materials and Methods), and the residual water-insoluble proteins were then solubilized by heating at 100°C for 5 min in 1% SDS and 5% 2-mercaptoethanol. The proteins were separated by SDS PAGE and transferred electrophoretically to nitrocellulose paper. The same blot was stained sequentially with Fast green, AE1, and AE3. (*a*) Fast green staining. Lanes *1* and *2* represent the innermost epidermal sections, and lanes *8–13* represent the thick s. corneum of heel epidermis. Keratins from living layers of abdominal epidermis (*A*; see Fig. 2, lane *1*) are shown on the right side for comparison. (*b*) AE1 staining. Picture was taken through a green filter so that only the brown PAP immuno-reaction product was visible. Note that the 50,000-mol wt keratin is present in basal and all upper layers, and that the 56,000-mol wt keratin is detectable only in upper cell layers. (*C*) AE3 staining. Note the detection of the 58,000-mol wt keratin in the innermost basal cells. The 65–67,000-mol wt keratins are weakly stained in lane *1*, but strongly stained in all other fractions. Molecular equivalents at left are × 10^{-3}.

bodies to human epidermal keratins. Since these antibodies are highly specific for keratins, they are useful for defining and detecting epidermal keratins and related molecules (51). In the present study, we used these antibodies to investigate the expression of keratin antigens during epidermal differentiation. The results are summarized schematically in Fig. 8.

65- to 67-kd Keratins

Previous results from conventional antisera showed that the 65- to 67-kd keratins are present only in cells above the basal layer (44, 52, 53; cf. 14). Our observation that AE2, which was specific for the 65- to 67-kd keratins and a 56-kd keratin, stained only suprabasal cells (Fig. 5) supports this conclusion.

58-kd Keratin

The observation that AE3 antibody, which recognized both the 58-kd keratin and the suprabasally located 65- to 67-kd keratins stained the entire epidermis (Fig. 6) suggested that the 58-kd keratin was present in basal cells. This was confirmed by the detection of the 58-kd keratin in horizontal skin sections containing predominantly epidermal basal cells (Fig. 7 *c*, lanes *1* and *2*). Our findings, in conjunction with some earlier [^{35}S]-methionine incorporation data (14), strongly suggested that the 58-kd keratin was made in basal cells and was retained as cells left the basal layer.

The interpretation that AE3 reacted with a suprabasally located 65- to 67-kd keratin triplet and a uniformly distributed 58-kd keratin is also consistent with the observation that suprabasal cells occasionally stained stronger than basal cells (Fig. 6 *b*).

56-kd Keratin

Immunoblot analysis of keratins isolated from horizontal sections of the epidermis indicated that the 56-kd keratin was not detectable in basal cells (Fig. 7 *b*). Although the precise location of this keratin has not yet been determined, the resolution between sections enriched with basal cells and those containing the 56-kd keratin strongly suggested that this keratin was not present in basal cells or cells immediately above the basal layer. This interpretation is supported by some earlier data that a 56.5-kd keratin which probably corresponds to the 56-kd keratin described here, is synthesized only late during keratinization (14).

The observation that AE1 failed to stain any cells above the basal layer (Fig. 4) suggested that the 56-kd keratin was at least

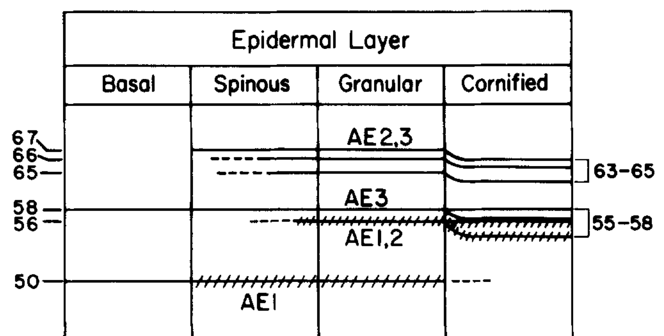

FIGURE 8 Schematic summary of changes in keratin during normal epidermal differentiation. A solid line denotes the presence of the keratin and a dotted line indicates the possible presence of the keratin in the cell layer. A hatched line indicates that the antigen was present but undetectable by AE1 antibody in frozen sections. Breaks in lines between granular and cornified layers indicate partial degradation of keratins during stratum corneum formation. Molecular equivalents at left are × 10^{-3}.

partially masked *in situ*. Although the suprabasal staining of the epidermis produced by AE2 antibody (Fig. 5) was consistent with the assignment of the 56-kd keratin to cells above the basal layer, it was impossible to determine from the present data whether in frozen sections AE2 detected both the 56-kd keratin and the 65- to 67-kd keratins, or only the high molecular weight keratin triplet.

Taken together, the results indicated that a 56-kd keratin, as defined by AE1 and AE2 antibodies, was absent in the basal layer and was probably made (but at least partially masked) in the upper spinous layer (Fig. 7 *b*). During s. corneum formation, this keratin gave rise to a 55-kd keratin, possibly through limited proteolysis (Figs. 2 and 7 *b*).

50-kd Keratin

The staining of epidermal basal layer by AE1 (Fig. 4) established that at least one of the two keratins (50 or 56-kdaltons) specified by this antibody must be present in basal cells. Immunoblot analysis of keratins extracted from horizontal sections (Fig. 7 *b*) clearly showed that it was the 50-kd keratin which existed in the basal layer. Although AE1 failed to stain cells above the basal compartment, direct analysis of keratins showed that the 50-kd keratin persisted in suprabasal cells (Figs. 7 *b* and 8).

Furthermore, our results indicated that, depending on the anatomic location of the epidermis, the 50-kd keratin underwent different degrees of proteolysis during keratinization. In abdominal epidermis, this keratin was almost completely eliminated during s. corneum formation (Fig. 2, lanes *2* and *5*). In heel epidermis, the elimination of this keratin was less complete (Fig. 7 *a* and *b*). Large quantities of this keratin were also detected in callus derived from several body sites, suggesting that the incomplete removal of this keratin might be a general phenomenon associated with the formation of a thick cornified layer or perhaps, more fundamentally, with a hyperproliferative state of the epidermis.

Masking of Keratin Antigens In Situ

Although direct analysis of keratins showed that the 50-kd keratin was present throughout epidermal living layers (Fig. 7 *b*), in frozen skin sections this keratin was detected by AE1 only in basal layer (Fig. 4; also see reference 3). Such results strongly suggested that the AE1 antigenic determinant on the 50-kd keratin was masked *in situ* in cells above the basal layer. Similarly, AE1 antibody failed to detect 56-kd keratin in frozen sections (Fig. 4). It is obvious from such results and from earlier reports on the *in situ* masking of certain keratin antigens (10, 16, 41) that keratins can best be localized by a combination of immunohistochemical staining of frozen sections and direct analysis of keratins. Immunohistochemical staining data alone (3, 52, 53) can be misleading and should be interpreted with caution.

Possible Significance of Keratin Heterogeneity

Our results indicated that only two major epidermal keratins (50 and 58-kd) were detected in basal cells of normal human epidermis. This finding is in accordance with the requirement of at least two keratin species for filament formation (26, 29, 42), and suggests the possibility that these two keratins may copolymerize to form filaments in basal cells. In addition, although the basal layer is known to contain a mixed population of stem cells and postmitotic cells (5, 25, 33), the observation that AE1 and AE3 antibodies stained the basal layers uniformly suggested that the expression of at least certain keratin antigens by basal cells was related to the histological (basal) location, rather than replicative potential, of epidermal keratinocytes.

The 65- to 67-kd keratins were recognized as a group immediately above the basal layer (14, 44, 52, 53). Although it is impossible to determine from immunological data whether all keratins in this group are expressed simultaneously, biochemical analysis by Fuchs and Green (14) suggests that the 67-kd keratin appears before other members of the triplet. The 56-kd keratin appeared to be synthesized somewhat later than the 65- to 67-kd keratins, probably in the upper spinous layers. These keratins may take part in forming new filaments; alternatively, they may bind to preexisting filaments. The latter possibility is particularly attractive as a possible mechanism by which the 50-kd keratin may be masked *in situ* in suprabasal cells, perhaps by the 65- to 67-kd keratins (Fig. 4).

The 56 and 65- to 67-kd keratins are made only by terminally differentiating cells of the in vivo normal epidermis and thus may be regarded as molecular markers of keratinization (cf. 8, 15, 17). This concept is supported by our recent finding that these keratins are normally absent in various nonkeratinized epithelia but appear when such epithelia are induced to keratinize (51; Tseng et al. Manuscript in preparation.). How the synthesis of these keratins may be regulated during keratinization is an important question which requires further investigation.

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