Acidic and Basic Hair/Nail ("Hard") Keratins: Their Colocalization in Upper Cortical and Cuticle Cells of the Human Hair Follicle and Their Relationship to "Soft" Keratins

Marion H. Lynch, W. Michael O'Guin, Carolyn Hardy, Linda Mak, and Tung-Tien Sun

Departments of Dermatology and Pharmacology and Kaplan Cancer Center, New York University School of Medicine, New York 10016

Abstract. Although numerous hair proteins have been studied biochemically and many have been sequenced, relatively little is known about their in situ distribution and differential expression in the hair follicle. To study this problem, we have prepared several mouse monoclonal antibodies that recognize different classes of human hair proteins. Our AE14 antibody recognizes a group of 10–25K hair proteins which most likely corresponds to the high sulfur proteins, our AE12 and AE13 antibodies define a doublet of 44K/46K proteins which are relatively acidic and correspond to the type I low sulfur keratins, and our previously described AE3 antibody recognizes a triplet of 56K/59K/60K proteins which are relatively basic and correspond to the type II low sulfur keratins. Using these and other immunological probes, we demonstrate the following. (*a*) The acidic 44K/46K and basic 56–60K hair keratins appear coordinately in upper corticle and cuticle cells. (*b*) The 10–25K, AE14-reactive antigens are expressed only later in more matured corticle cells that are in the upper elongation zone, but these antigens are absent from cuticle cells. (*c*) The 10-nm filaments of the inner root sheath cells fail to react with any of our monoclonal antibodies and are therefore immunologically distinguishable from the cortex and cuticle filaments. (*d*) Nail plate contains 10–20% soft keratins in addition to large amounts of hair keratins; these soft keratins have been identified as the 50K/58K and 48K/56K keratin pairs. Taken together, these results suggest that the precursor cells of hair cortex and nail plate share a major pathway of epithelial differentiation, and that the acidic 44K/46K and basic 56–60K hard keratins represent a co-expressed keratin pair which can serve as a marker for hair/nail-type epithelial differentiation.

HAIR originates from a follicular structure which consists of three parts: an outer root sheath, an inner root sheath that includes Henle's and Huxley's layers and the inner root sheath cuticle, and the hair fiber itself that includes the hair cuticle, cortex, and medulla (Montagna and Parakkal, 1974; Swift, 1977). The emerged hair fiber is composed primarily of cortical cells filled with 10-nm keratin filaments. Biochemical studies of wool filaments and their associated matrix material have led to the realization that there are three major classes of hair proteins: the "low sulfur keratins" (designated 40–60K), the "high-sulfur proteins" (10–25K), and the "high glycine/tyrosine proteins" (6–9K). It is believed that the low sulfur keratins form the 10-nm filaments and the latter two groups of proteins form interfilamentous matrix. More detailed analyses have shown that each of these three classes of hair protein can be further divided into subclasses (Fraser et al., 1972; Crewther, 1976; Gillespie, 1983; Rogers, 1984). The low sulfur keratins of wool have been resolved into multiple components (Nos. 5, 7, and 8) which fall into two subfamilies (type I and type II) according to their amino acid sequence homology to the two prototypic wool keratin fragments isolated and sequenced by Crewther and co-workers (Crewther, 1976; Crewther et al., 1976, 1978, 1980; Gough et al., 1978). The high sulfur proteins of wool can be resolved into 60–70 minor components, which can be grouped into at least three major subfamilies with molecular weights of 11,000, 16,000, and 19,000 (Crewther, 1976; Swart et al., 1976; Lindley, 1977; Powell et al., 1983). Finally, the high glycine/tyrosine proteins, of which over 20 have been identified so far, can be divided into one group of proteins which are poor in cystine but rich in phenylalanine, and another group for which the reverse is true (Zahn et al., 1980; Gillespie, 1983; Rogers, 1984).

The low sulfur keratins of hair and nail ("hard" keratins)[1]

1. In this paper, we use the terms "hard" and "soft" keratins, in a classical sense, to describe the alpha-keratins of the hardened, cornified tissues (e.g., hair and nail) and those of the "soft", mucosa-type epithelia, respectively (Fraser and Macrae, 1980).

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and the keratin intermediate filament of various "soft" epithelia including the epidermis are clearly related because (*a*) they share a similar x-ray diffraction pattern arising from alpha-helices in a coiled-coil conformation (Fraser et al., 1972; Baden et al., 1973); (*b*) all soft keratins can be divided by hybrid selection and immunological criteria into a basic and an acidic subfamily corresponding to the type I and type II wool low sulfur keratins, respectively (Fuchs et al., 1981; Schiller et al., 1982; Tseng et al., 1982; Hanukoglu and Fuchs, 1983; Kim et al., 1983; Eichner et al., 1984; Sun et al., 1984, 1985; Quinlan et al., 1985); (*c*) an antiserum prepared against wool merokeratin (the protease-resistant, alpha-helical domain of wool keratins) cross-reacts strongly with keratin fibers of PtK2 and HeLa cells, as well as several mucosal epithelia (Weber et al., 1980); and (*d*) amino acid sequences of hard and epidermal keratins show extensive homology (Hanukoglu and Fuchs, 1982; Weber and Geisler, 1982, 1984).

On the other hand, data are also available pointing to some unique features of hard as compared with soft keratins. Firstly, Baden and co-workers (Lee et al., 1976; Baden et al., 1980) have reported that their antisera prepared against human hair keratins react strongly with hair and nail keratins but fail to react with epidermal (soft) keratins. Secondly, hard keratins, at least those of wool and human hair, are almost totally insoluble under conditions that solubilize soft keratins (e.g., 8–9 M urea, 1% 2-mercaptoethanol in 10–25 mM Tris-HCl, pH 7.4; O'Donnell and Thompson, 1964; Crewther, 1976). This insolubility of hard keratins had led to the common practice of solubilizing the proteins either by proteolytic digestion or by S-carboxymethylation after reduction in alkaline conditions (Crewther, 1976). Unfortunately, such covalent modifications have made it difficult to compare the biochemical and immunological properties of hard and soft keratins since the latter have been characterized without such modifications.

This tremendous heterogeneity in hair proteins, coupled with the complex histological features of the hair follicle, constituted a major obstacle to discerning the in situ distribution and function of hair proteins. Hence, despite the fact that over 25 wool proteins have been completely sequenced, relatively little is known concerning their association with specific hair structures or their differential expression during hair differentiation (reviewed in Gillespie, 1983; Rogers, 1984, 1985; Powell and Rogers, 1986).

In this paper, we describe the characterization of human hair and nail keratins (in an unmodified or "-SH form") by one- and two-dimensional gel electrophoresis followed by immunoblotting using a panel of monoclonal antibodies. Three of these monoclonal antibodies (AE12, AE13, and AE14) have been raised against human hair fiber proteins and are described for the first time here. Our results have established that hair and nail share a relatively basic, AE3-reactive, 56K/59K/60K (type II) keratin triplet and a relatively acidic, AE12- and AE13-reactive, 44K/46K (type I) keratin doublet. Immunolocalization data indicate that these basic and acidic keratins appear coordinately in upper cortical and cuticle cells of the hair follicle. Moreover, since hu-

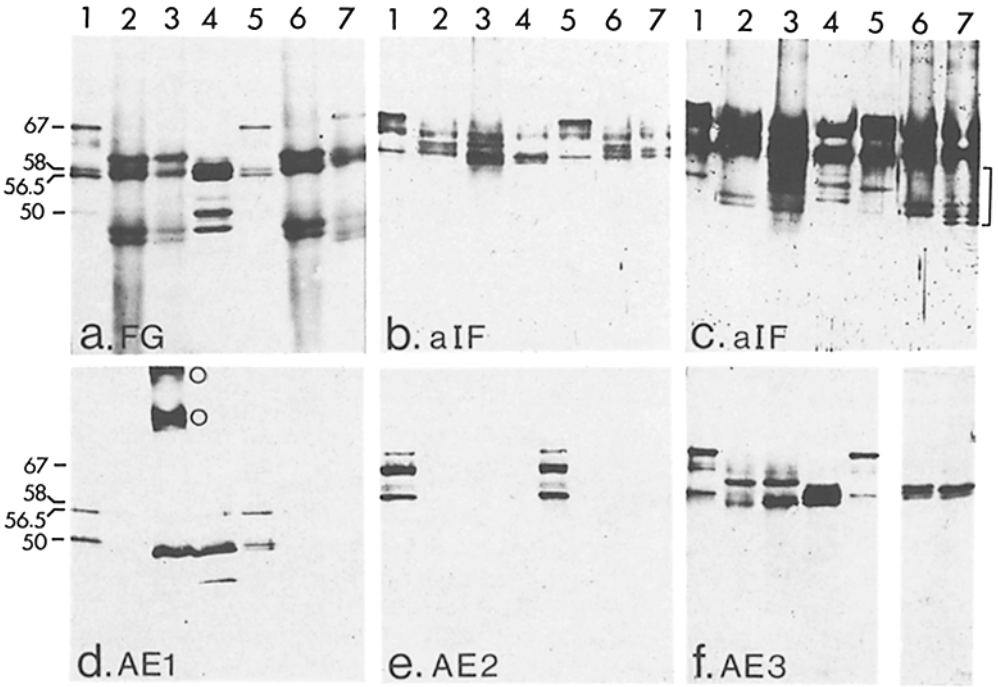

Figure 1. One-dimensional immunoblot analyses of human hair and nail keratins using aIF antibody as well as monoclonal antibodies raised against "soft" keratins. Keratins were extracted from (lanes *1* and *5*) human abdominal epidermis, (lanes *2* and *6*) human hair, (lane *3*) human nail plate (dorsal part only), (lane *4*) cultured human newborn foreskin epidermal cells, and (lane *7*) wool. The proteins were resolved by SDS PAGE on 12.5% gels, transferred electrophoretically to nitrocellulose, and stained with (*a*) Fast green, (*b*) aIF monoclonal antibody that recognizes all basic keratins strongly, and most acidic keratins weakly (Cooper et al., 1984), (*c*) same as in *b* but with a greater amount of samples loaded to show the weak immunoreactivities of the acidic keratins, (*d*) AE1, which recognizes many acidic (type I) keratins (Sun et al., 1984), (*e*) AE2, which recognizes the 56.5K and 65–67K markers for "skin-type" differentiation (Tseng et al., 1982; Woodcock-Mitchell et al., 1982; Cooper et al., 1985), and (*f*) AE3, which defines all known basic (type II) "soft" keratins (Sun et al., 1984). Note the overall similarities among hair, nail, and wool keratins, with the exception that nail contains AE1-positive 48K, 50K, and two high molecular weight bands (marked by circles in Fig. 1 *d*). Also note that the three largest hair keratins are AE3 positive. The AE3-positive 64K band in lanes *2* and *3* of *f* is probably due to stratum corneum contamination (from some chemical reagents) which was not detected in most of our other experiments (cf., lane *6* in the same panel; also see Figs. 3 *c* and 5 *c*; Ochs, 1983; Cooper et al., 1985).

man soft keratins have been well characterized (unlike those of the sheep), by concentrating on human hair and nail keratins we were able to compare several important aspects of soft and hard keratin expression. Such a comparison has allowed us to integrate the available information on these two types of keratins, and to conclude that the acidic and basic hard keratins may be regarded as another coexpressed pair (Sun et al., 1984; Cooper et al., 1985) that represents a marker for "hair/nail-type" of epithelial differentiation.

Materials and Methods

Keratin Extraction

Human hair clippings were minced to <2–3 mm in length, incubated under nitrogen in 8 M urea, 200 mM Tris-HCl (pH 9.5) and 200 mM 2-mercaptoethanol for 2 h, at which time the physically swollen hair was homogenized with a Polytron for 30 s, and the incubation was continued under nitrogen for an additional 2 h (O'Donnell and Thompson, 1964; Baden et al., 1973; Steinert and Rogers, 1973). Insoluble material was removed by centrifugation at 10,000 *g* for 10 min, and the supernatant was stored at −70°C in small aliquots until use. Hairs clipped from various points along mature hair shafts of three normal individuals have been used, yielding similar results.

Distal clippings of normal human nail plates were scraped to remove the ventral 1/4 portion in order to be certain that the specimen was free of contamination by epidermal stratum corneum which tends to accumulate under the nail. The nail clippings were subsequently filed into a fine powder and extracted by the same procedure as the hair. Similar results were obtained from samples derived from three individuals.

Keratins were extracted from fresh human epidermis and from cultured newborn human foreskin epidermal cells using 8 M urea, 25 mM Tris-HCl (pH 7.4), and 25 mM DDT as described previously (Woodcock-Mitchell et al., 1982).

To minimize proteolysis, all solutions contain a mixture of protease inhibitors as described (Woodcock-Mitchell et al., 1982).

Antibodies

The preparation and characterization of mouse monoclonal antibodies AE1, AE2, and AE3 have been described (Woodcock-Mitchell et al., 1982). The mouse monoclonal antibody (anti-intermediate filament [aIF]) recognizing a determinant common to all types of intermediate filaments (Pruss et al., 1981) is a gift from Dr. Rebecca Pruss of the National Institutes of Health. A polyclonal rabbit anti-laminin serum is a gift from Drs. Hinda Kleinman and George Martin of NIH (Timpl et al., 1979).

The AE12, AE13, and AE14 antibodies were prepared as follows: human hair keratins in 8 M urea were dialyzed against 0.1% SDS, 25 mM Tris-HCl, pH 7.4, and 100 mM 2-mercaptoethanol, emulsified with either complete (for primary immunization) or incomplete (for boosting) Freund's adjuvant, and injected subcutaneously into multiple sites in female BALB/c mice (200 μg protein per mouse). Spleen cells from these mice were fused with P3X63 Ag. 8.653 myeloma cells (kindly provided by Dr. M. Scharff of Albert Einstein Medical College) using 50% polyethylene glycol 1450 (Merck) according to Kohler and Milstein (1975). Hybridoma cells were grown in the presence of macrophage feeder cells, selected with hypoxanthine/aminopterin/thymidine (HAT) medium, and assayed for anti-keratin activities by ELISA (Woodcock-Mitchell et al., 1982). Colonies selectively reacting with hair proteins (but not with epidermal keratins) were cloned three times by limiting dilution (for some strategies useful in preparing monoclonal antibodies specific for individual keratins, see Cooper et al., 1985).

Gel Electrophoresis and Immunoblotting

One- and two-dimensional gel electrophoreses (both IEF and NEPHGE) were performed according to Laemmli (1970) and O'Farrell et al. (1975; 1977), respectively. Immunoblotting was done according to Towbin et al. (1979) with some modifications that facilitate the identification of individual keratins by recording photographically both the fast green and the peroxidase antiperoxidase immunoreaction staining patterns of the same blot (Woodcock-Mitchell et al., 1982; Eichner et al., 1984).

Immunofluorescence Staining

Frozen sections of human scalp (6 μm) were stained by indirect immunofluorescence as described previously (Sun and Green, 1978*b*; Sun et al., 1979).

Results

Human Hair Keratins

The solubilization of human hair keratins by 8 M urea con-

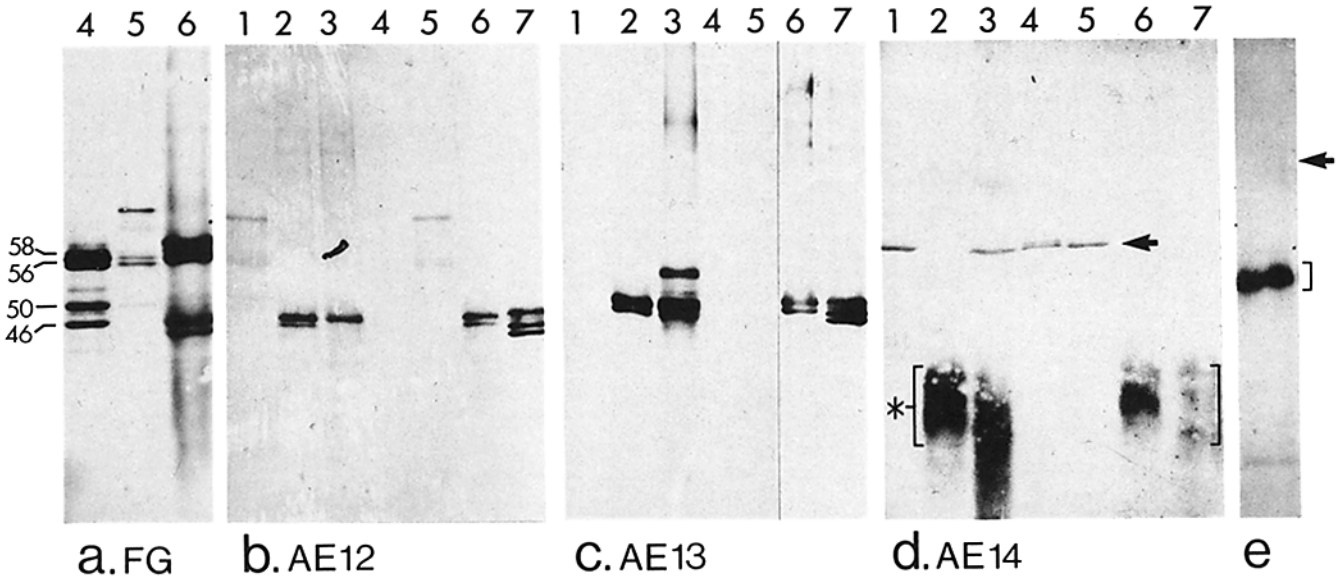

Figure 2. One-dimensional immunoblot analyses of human hair and nail keratins with monoclonal antibodies raised against hair keratins. The numbering of keratin samples is the same as in Fig. 1. The sample in *e* contains human hair keratins analyzed on a 20% (instead of the usual 12.5%) polyacrylamide gel. Gels were stained with (*a*) Fast Green, (*b*) AE12, (*c*) AE13, and (*d* and *e*) AE14. Note that both AE12 and AE13 recognize the 44K and 46K hair keratins. AE13 does not seem to cross-react with any "soft" keratins, while AE12 shows weak cross-reaction with the 56.5K/65-67K keratins. Also note that AE14 reacts with 10–25K material (marked with brackets in *d*) in hair, and a 58K (No. 5) keratin (arrow) in epidermis.

taining a reducing agent is much more efficient at pH 9.5 than at neutral pH routinely used for the extraction of soft keratins. There are two possible explanations for the influence of pH: (*a*) disulfide bonds, which are much more abundant in hard keratins than in soft ones, are reduced more efficiently under alkaline conditions; and (*b*) high pH may induce partial unfolding of the otherwise compact keratin structure, making the disulfide bonds more easily accessible to reducing agents.

One-dimensional SDS PAGE showed that among the human hair keratins solubilized under our conditions we can resolve five major bands: a triplet of 56,000, 59,000, and 60,000, designated 56K/59K/60K, and a doublet of 44,000 and 46,000, designated 44K/46K (Fig. 1 *a*, lane *2*; Fig. 2 *a*, lane *6*). Two-dimensional PAGE showed that the 56K/59K/60K components are relatively basic (pI 6.4–6.7) and the 44K/46K components are relatively acidic (pI <5; Fig. 3). Since most of the hair keratins (except the 60K component) have a pI of <6.5, better resolution can be obtained for these proteins using the IEF–SDS gel system which was, therefore, used in most of our subsequent analyses.

Electrophoretic and immunoblotting data showed that the triplet and the doublet belong to two distinct classes: the relatively basic 56K, 59K, and 60K components react strongly with the aIF antibody (Fig. 1 *b*, lane *2*, and Fig. 4 *b*). This is consistent with our previous finding that the aIF antibody reacts preferentially with the larger keratins that belong to the basic subfamily (Cooper et al., 1984). In addition, these hair keratins react strongly with our AE3 antibody which recognizes all known soft keratins of the basic, type II subfamily (Fig. 1 *f*, lanes *2* and *6*; Fig. 4 *c*; see Sun et al., 1984; Cooper et al., 1985). Taken together, these results suggest that the human 56K/59K/60K triplet corresponds to the low sulfur wool keratins Nos. 5 and 7c (reported molecular weights of 55,000–58,000; Woods, 1979), and belong to the basic, type II keratin subfamily (Crewther, 1976, 1980; also see below).

The acidic 44K and the 46K components react, although only weakly, with the aIF antibody which has been shown previously to recognize all intermediate filament proteins including all soft keratins (Fig. 1 *c*, lane *2*; for two-dimensional immunoblot, see Figs. 4, *a* and *b*; Pruss et al., 1980; Cooper et al., 1984), thus confirming their homology with intermediate filament proteins. These two components do not react with our AE1 antibody which recognizes many soft keratins of the acidic subfamily (Fig. 1 *d*, lanes *2* and *6*; see Tseng et al., 1982; Sun et al., 1984; Cooper et al., 1985). However, these two proteins react strongly with two of our new monoclonal antibodies raised against human hair keratins (for results obtained with AE12 antibody see Fig. 2 *b*, lanes *2* and *6*; for AE13 results, see Fig. 2 *c*, lanes *2* and *6*). The fact that AE13 did not cross-react with any soft keratins (Fig. 2 *c* and data not shown) suggests that these acidic 44K/46K hair keratins possess unique epitopes. The relative charge and size (and the corresponding two-dimensional gel mobility) of the 44K/46K hair proteins strongly suggest that they correspond to the previously characterized low sulfur sheep wool keratin No. 8 (reported molecular weight of 45,000–50,000; O'Donnell and Thompson, 1964; Woods, 1979), and belong to the acidic, type I keratin subfamily (Crewther, 1976, 1980).

In addition to these five major intermediate filament-related proteins, human hair contains a small amount of low molecular weight material which, although barely detectable by Fast Green or Coomassie Blue (Fig. 1 *a*, lanes *2* and *6*; and Fig. 2 *a*, lane *6*), can be visualized readily with another new monoclonal antibody, AE14, as a 10–25K smear (Fig. 2 *d*, bracketed zones in lanes *2* and *6*). This AE14-reactive material appears as a much better defined "band" when the acrylamide concentration of the gel is increased from 12.5% to 20% (Fig. 2 *e*). It is present in all hard keratin preparations that we have examined so far including human nails (Fig. 2 *d*, lane *3*) and sheep wool (Fig. 2 *d*, lane *7*; also see below), but is not detectable in the epidermis (Fig. 2 *d*, lanes *1* and *5*). The size range and species distribution of this material suggest that it may correspond to at least some of the so-called high sulfur proteins. The high glycine/tyrosine proteins are much smaller in size (6–9K) and are reported to be absent from human hair (Fraser and MacRae, 1982; Gillespie, 1983; Marshall, 1983).

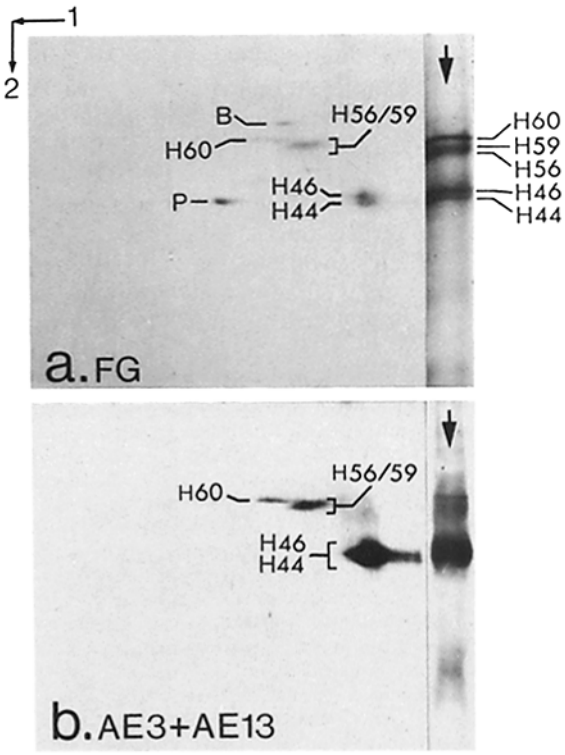

Figure 3. Two-dimensional polyacrylamide gel analysis of human hair keratins. *1* and *2* denote the directions of the first dimensional, nonequilibrium pH gradient electrophoresis and second dimensional SDS PAGE, respectively. After electrophoresis the proteins were transferred onto nitrocellulose and stained first with (*a*) Fast green, and then with (*b*) a mixture of AE3 (which recognizes the 56K/59K/60k keratins) and AE13 (44K/46K) monoclonal anti-keratin antibodies. Note the resolution of hair keratins into two distinct groups. For a comparison of the one- and two-dimensional gel patterns, the sample was also applied to a side lane (on the right, marked by a downward arrow) during the second dimensional SDS PAGE. *B* and *P* are BSA and 3-phosphoglycerate kinase markers, respectively.

Human Nail Keratins

To compare the keratins of the hair with those of another hard tissue, we analyzed keratins of human nail plate. Con-

sistent with previous studies (Baden et al., 1973; Marshall, 1983), we found that the major nail polypeptides (Fig. 1 *a*, lane *3*) are similar to those of the hair (Fig. 1 *a*, lanes *2* and *6*) both in electrophoretic mobility and in immunoreactivity with various monoclonal anti-keratin antibodies (Figs. 1, 2, and 5).

We also found that nail plate contains a small but significant amount (10–20% as estimated by Fast green staining; Figs. 1 *a* and 5 *a*) of soft keratins that we have identified as the 48K, 50K, 56K, and 58K keratins (for the tissue distribution and immunological properties of the soft keratins, see Moll et al., 1982; Sun et al., 1984). Figs. 1 *d* (lane *3*) and 5 *d* show the immunological detection of the acidic 50K and 48K keratins in nail by AE1 blotting. The identity of these two keratins was confirmed by mixing keratin preparations from the nail plate and from cultured human epidermal cells, and then performing co-electrophoresis by two-dimensional PAGE (Fig. 5 *f*). The presence of the basic 58K and 56K keratins in nail was established by two-dimensional gel electrophoresis in which the first dimensional separation was done under nonequilibrium conditions (data not shown). The presence of the 58K keratin in nail was also confirmed by immunoblotting using the AE14 antibody which recognizes, in addition to the 10–25K high sulfur hair proteins, the 58K keratin of the epidermis (Fig. 2 *d*, lane *3*).

Nail, like hair, did not react with AE2 antibody which recognizes the 56.5K and 65–67K keratin markers of the ("soft-type")-"keratinization" or "skin-type" differentiation (Fig. 1 *e*, lanes *2* and *3*; Woodcock-Mitchell, 1982; Tseng

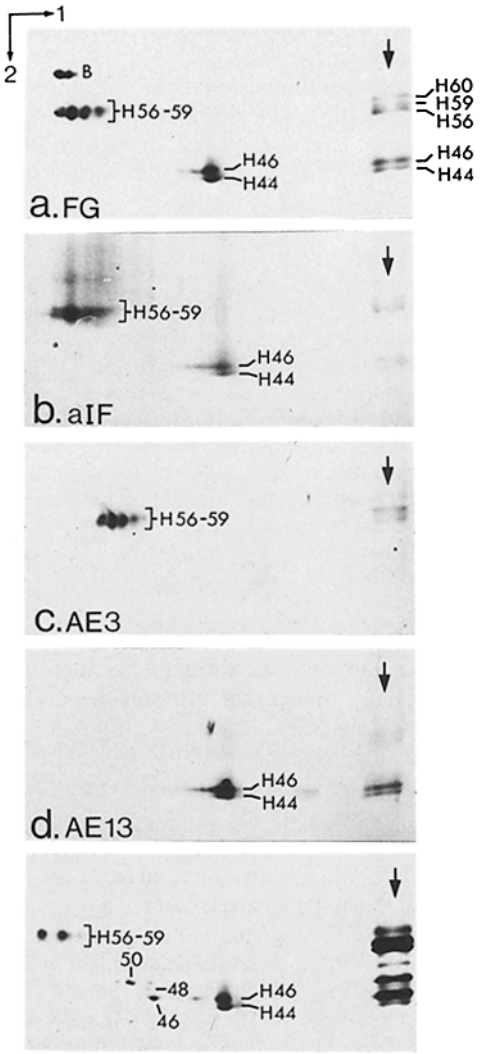

Figure 4. Two-dimensional immunoblot analyses of human hair keratins. *1* and *2* denote the directions of the first dimensional IEF and second dimensional SDS PAGE, respectively. Basic proteins are on the left. Note the improved resolution of most hair keratins as compared to the NEPHGE SDS gel system (Fig. 3), and the absence of 60K keratin which is too basic to remain during IEF. (*a* and *e*) Fast-green, (*b*) aIF, (*c*) AE3, and (*d*) AE13. The sample in *e* is a mixture of keratins from the hair and cultured human epidermal cells. BSA (labeled *B* in panel *a*) was added as a marker.

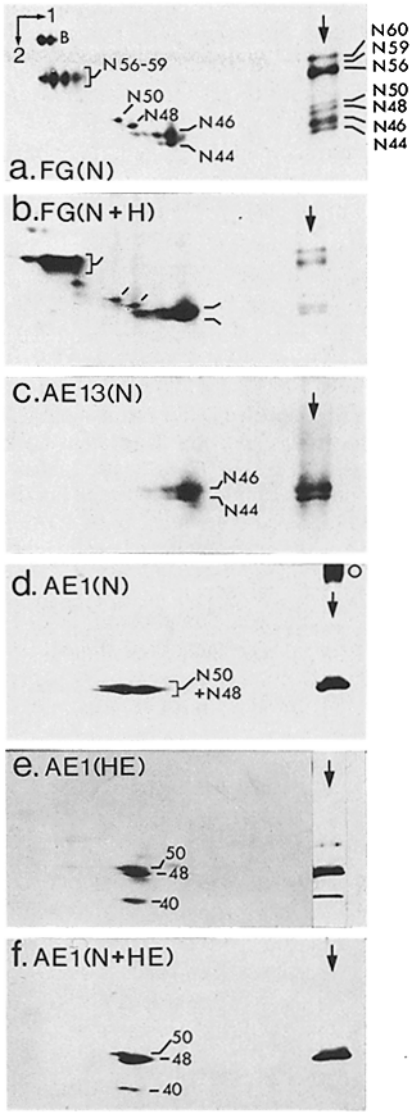

Figure 5. Two-dimensional immunoblot analyses of human nail keratins. (*a* and *b*) Fast green, (*c*) AE13, and (*d–f*) AE1. Samples are (*a*, *c*, and *d*) nail (*N*); (*b*) a mixture of nail keratins plus hair (*H*) keratins, showing that the major components are nearly identical; (*e*) keratins of cultured human epidermal (*HE*) cells; (*f*) a mixture of keratins from nail and cultured human epidermal cells. Note in *f* that the minor nail components (labeled N50 and N48 in *a*) co-migrate with the 50K and 48K soft keratins of cultured human epidermal keratinocytes.

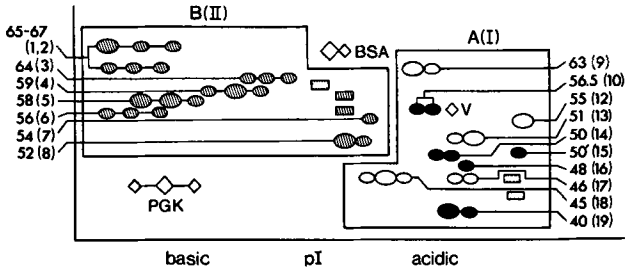

Figure 6. A schematic diagram of the two-dimensional electrophoretic pattern of human keratins (a composite of IEF- and NEPHGE SDS gel patterns). Keratins are identified by molecular weights ($\times 10^{-3}$) as well as by catalogue numbers (in parentheses; Moll et al., 1982). They are divided into the acidic (type I) and basic (type II) subfamilies, according to their relative charges and immunoreactivities. Hard keratins as described in the present work are represented by rectangles (H56, H59, H60 of the basic subfamily, and H44 and H46 of the acidic subfamily). Note that all known basic keratins, including those of the hair, are recognized by AE3 (*hatched ovals and rectangles*), while many but not all acidic keratins are recognized by AE1 (*solid circles*). H44 and H46 keratins are recognized by AE13 (*dotted rectangles*). *A (I)*, acidic type I keratins; *B (II)*, basic type II; *pI*, isoelectric point; *V*, vimentin; *BSA*, bovine serum albumin; and *PGK*, 3-phosphoglycerate kinase.

et al., 1982, 1984; Cooper et al., 1985). The lack of these two epidermal keratins in nail plate proves that the "soft" keratins that we have detected in nail cannot be due to stratum corneum contamination.

Although AE12 and AE13 gave very similar reaction patterns with human nail keratins, they differ mainly in that AE13 sometimes detects an additional, minor, 52K nail component (undetectable by Fast green staining) not seen in hair (Fig. 2 *c*, lane *3*; Marshall, 1983). Whether or not this component is truly nail specific will require further investigation.

Several high molecular weight (100–200K), keratin-related species can be detected in nail by immunoblotting. They react strongly with AE1 (Fig. 1 *d*, lane *3*, marked with circles), but weakly with AE3 (Fig. 1 *f*, lane *3*) and AE13 (Fig. 2 *c*, lane *3*). Although the significance of these high molecular weight bands is not yet clear, they are stable even during heating in the presence of SDS and fresh reducing agent, suggesting that keratins may be cross-linked (either with other keratins or with nonkeratin proteins) through nondisulfide, covalent bonds.

Fig. 6 summarizes the two-dimensional gel mobilities of known human epithelial keratins including those of the hair and nail.

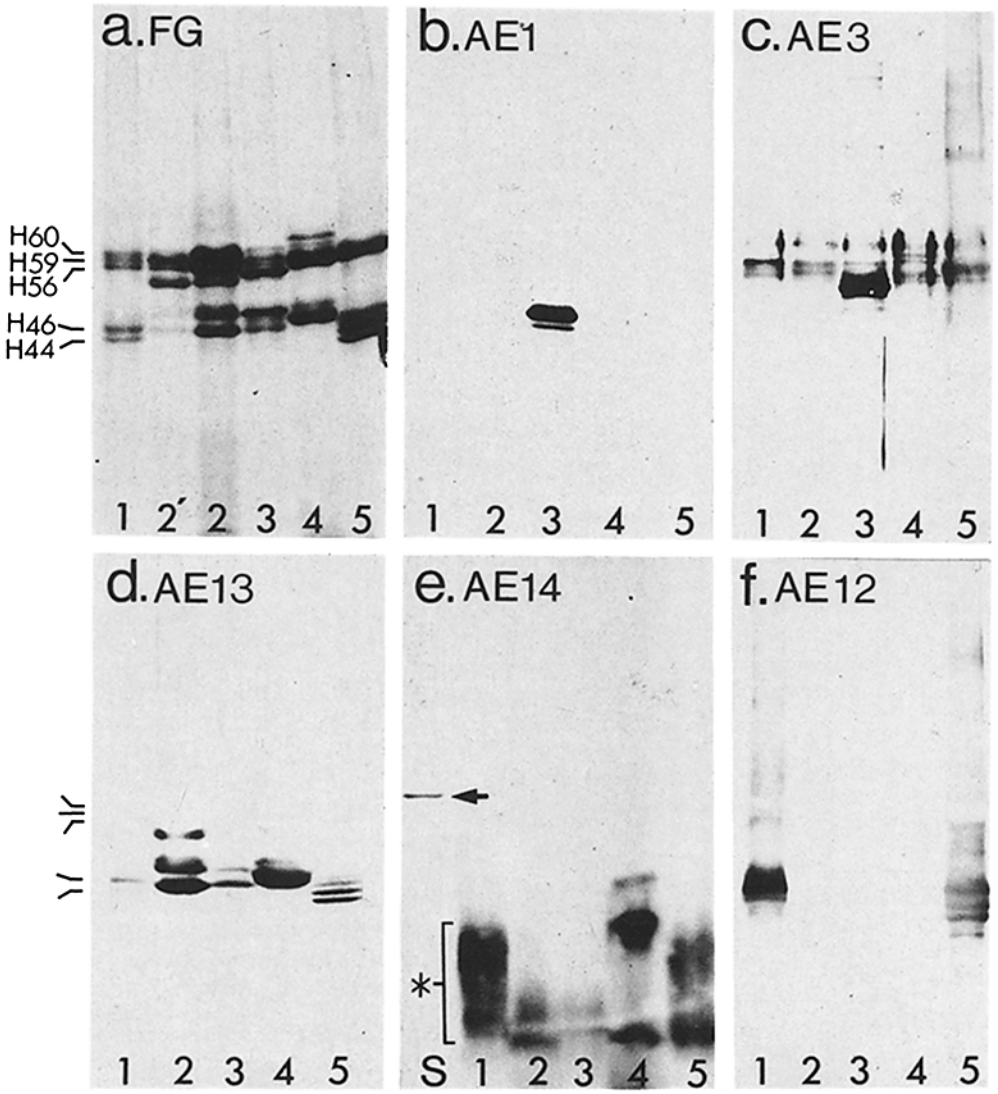

Figure 7. One-dimensional immunoblot analyses of hair keratins from several species. All samples except lane 2′ of *a* were extracted at pH 9.5 (see Materials and Methods). (Lanes *1*) human hair; (lane *2′*) rabbit hair extracted with 8 M urea, 25 mM Tris-HCl (pH 7.4), 10 mM dithiothreitol; (lanes *2*) rabbit hair; (lanes *3*) rabbit claw; (lanes *4*) mouse hair; and (lanes *5*) wool. The proteins were transferred to nitrocellulose and stained with (*a*) Fast green, (*b*) AE1, (*c*) AE3, (*d*) AE13, (*e*) AE14, and (*f*) AE12. The first lane in *e*, labeled *S*, is a sample of human skin epidermal keratin showing the cross-reaction of AE14 antibody with the 58K soft keratin (marked by an arrow). Asterisk denotes the 10–25K, AE14-positive material. Note the similar size distribution and AE3 and AE13 immunoreactivities of the hair samples from different species.

Hair and Nail Equivalents of Other Species

One- and two-dimensional immunoblot analyses of the hair proteins from rabbit (Fig. 7 *a*, lane *2*), mouse (lane *4*), and sheep (wool; lane *5*) demonstrated that they are strikingly similar to human hair keratins (lane *1*), not only in their overall size (Fig. 7 *a*) and charge (for a comparison of wool and human hair keratins by two-dimensional gels, see Fig. 8), but also in their immunoreactivities with AE3 (Fig. 7 *c*), AE13 (Fig. 7 *d*), and AE14 antibodies (Fig. 7 *e*). The presence of 50K/58K and 48K/56K soft keratins in nail was also confirmed using rabbit claws (Figs. 7, *b* and *c*, lane *3*). In the meantime, our results indicate that there exist some species variations. For example, while AE13 reacts strongly with the 44–46K, acidic, hard keratins in all species examined (Fig. 7 d), AE12 reacts well only with human and sheep antigens (Fig. 7 *f*), suggesting that the two antibodies recognize distinct epitopes. Another species variation is that, unlike human hair keratins which can only be effectively solubilized after prolonged extraction in 8 M urea at high pH, a significant amount of the 56–60 K, basic keratins can be solubilized from mature rabbit hair after incubation in 8 M urea, 25 mM Tris-HCl (pH 7.4), plus 100 mM 2-mercapto-

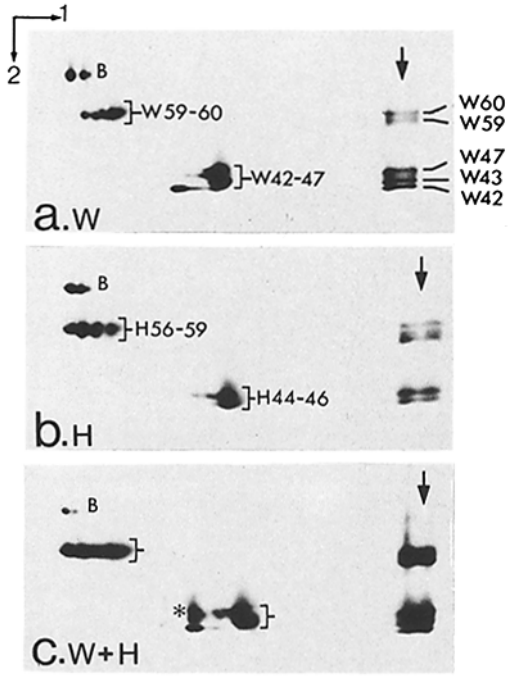

Figure 8. Two-dimensional polyacrylamide gel electrophoresis of keratins from (*a*) sheep wool, (*b*) human hair, and (*c*) a mixture of human hair and wool. Symbols are identical to those of Figs. 4 and 5; asterisk denotes the shift of some acidic keratin toward the basic end, possibly due to complex formation (Franke et al., 1983). Note the striking similarity between human hair and sheep wool keratins.

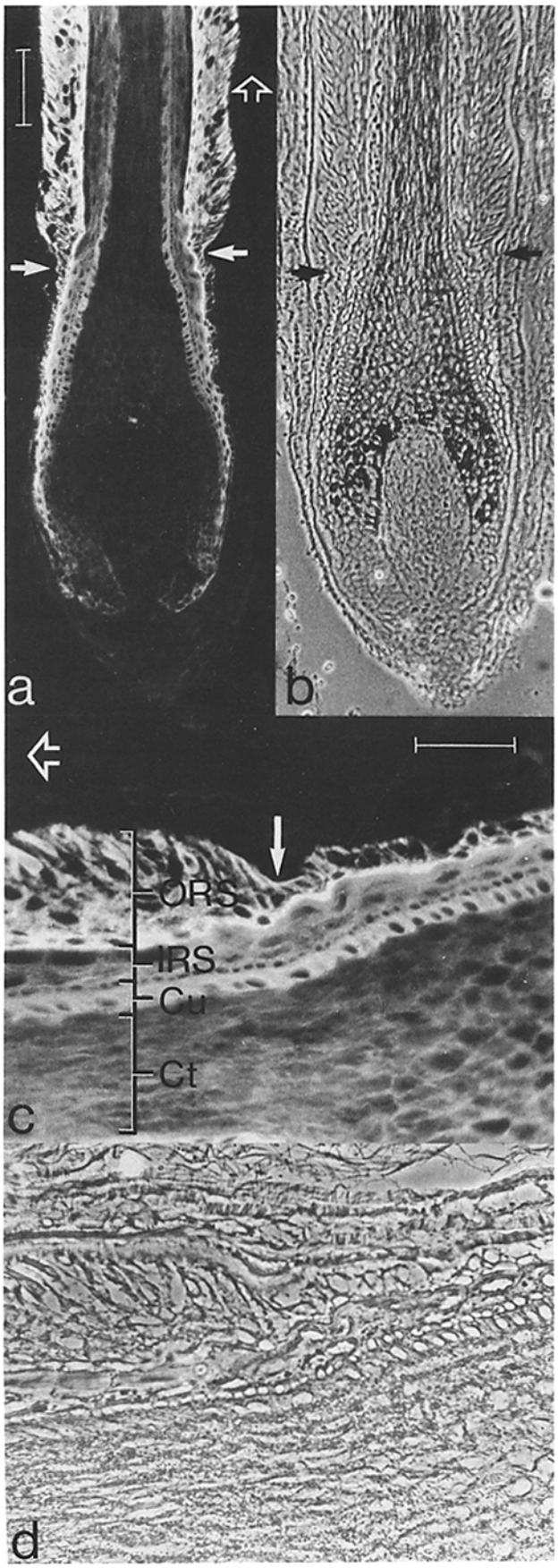

Figure 9. Indirect immunofluorescent staining of a human scalp hair follicle with a rabbit antiserum to total human epidermal (callus) keratins. *a* and *c* are fluorescence pictures, and *b* and *d* are their corresponding phase-contrast images. Solid arrows denote a zone where the outer root sheath undergoes an abrupt transition from very thin (2–4 layers of cells) to become much thicker (>6 layers). *ORS*, outer root sheath; IRS, inner root sheath; *Cu*, hair cuticle, and *Ct*, cortex. The large empty arrows in *a* and *c* point to the direction of hair growth. Note the strong staining of outer and inner root sheaths as well as hair cuticle cells, and the relatively weak staining of cortical cells. Bars, (*a* and *b*) 100 μm; (*c* and *d*) 50 μm.

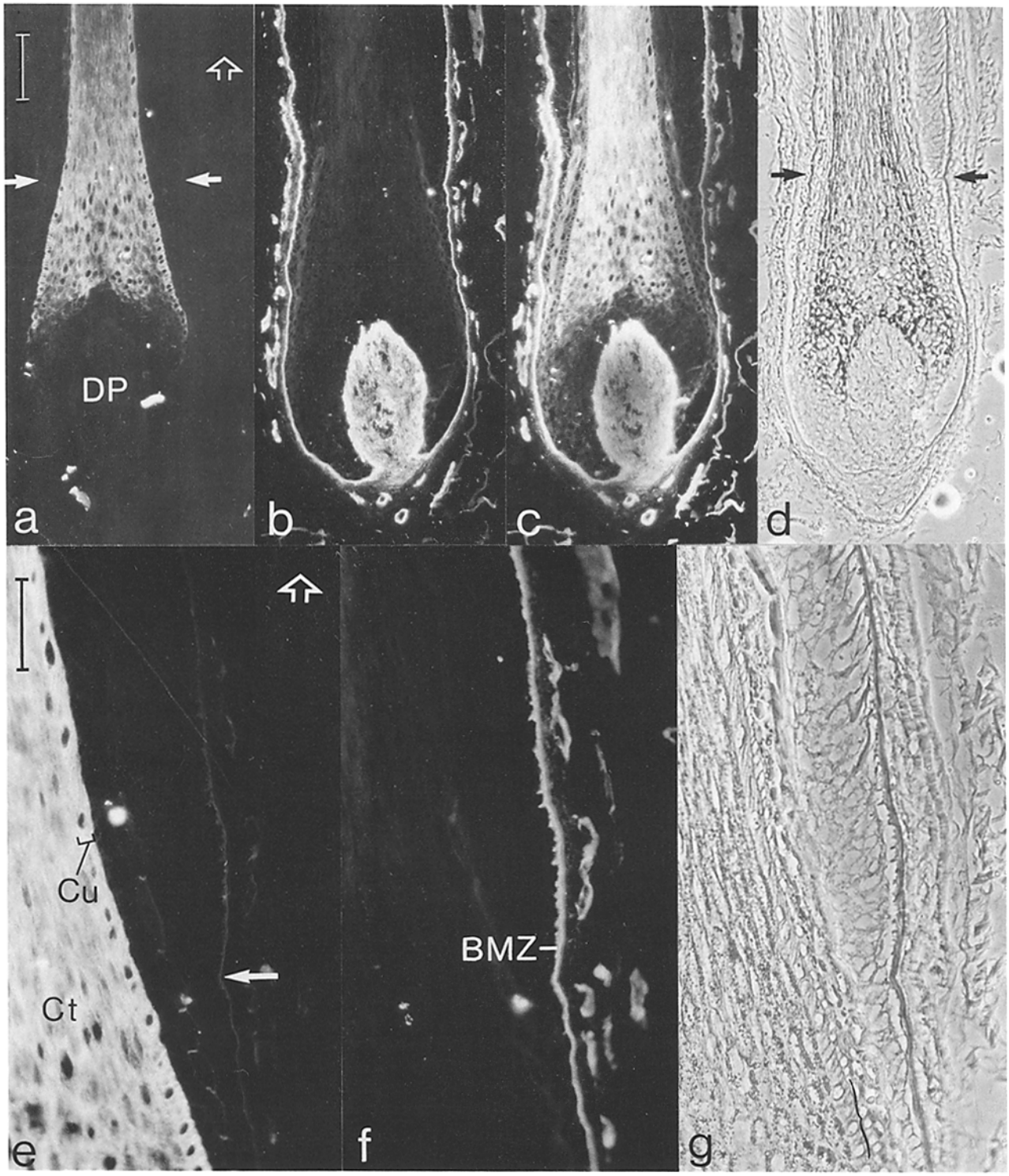

Figure 10. Double immunofluorescent staining of a human scalp hair follicle using AE13 mouse monoclonal antibody specific for the acidic 44/46K low sulfur hair keratins and polyclonal rabbit antiserum to laminin. (*a–d*) show the same field stained with (*a*) AE13, (*b*) rabbit anti-laminin to delineate the basement membrane zone, (*c*) a double exposure showing both AE13 and laminin staining, and (*d*) phase-contrast image. *e–g* show the same field stained with (*e*) AE13, (*f*) rabbit anti-laminin, and (*g*) phase-contrast. *DP*, dermal papilla; *BMZ*, basement membrane zone; other symbols are the same as in Fig. 9. Note the strong AE13 staining of upper cortical and hair cuticle cells. Bars, (*a–d*) 100 μm; (*e* and *f*) 50 μm.

ethanol (Fig. 7 *a*, lane 2′). Although the structural meaning of this finding is not yet apparent, it raises the cautionary note that epidermal keratins extracted from some rodent skins using the standard "urea/reducing agent/neutral pH" protocol could be contaminated by some basic keratins from the hair follicles.

Immunolocalization of Hard Keratins in Human Hair Follicle

To examine the expression of human hair keratins in various parts of the follicle and in different stages of follicular differentiation, we performed indirect immunofluorescence staining on frozen sections of human scalp skin. Fig. 9, *a* and

c, shows the staining patterns produced by a polyclonal rabbit antiserum raised against total human epidermal keratins (Sun and Green, 1978, *a* and *b*). This antiserum was not found to cross-react with the hair keratins by immunodiffusion (O'Guin et al., 1986). However, strong staining can be seen in both the outer and inner root sheath cells, as well as in the cuticle cells of the hair shaft. On the other hand, cortex cells, particularly those located in the deep bulbar region, are stained extremely weakly, if at all (Fig. 9, *a* and *c*).

The antibodies AE13 (specific for the acidic hair keratins) and AE3 (which reacts with the basic hair keratins) both stain intensely the upper cortex and hair cuticle cells (Figs. 10, *a* and *e*, and 11, *a* and *c*). This staining is first seen ~5–10 cell layers above the top of the dermal papillae, in an area corresponding to the beginning of "elongation zone" of the follicle (Kemp and Rogers, 1970; Swift, 1977; Orwin, 1979). That multiple layers of deep bulbar cells indeed do not react with AE13 was confirmed by double-staining using both mouse AE13 and a rabbit antiserum against laminin which delineates the basement membrane zone (Fig. 10, *a–c*). Since AE3 cross-reacts with all known keratins of the basic subfamily, this antibody also stains the soft keratin–containing outer root sheath (Fig. 11, *a* and *c*). The inner root sheath layers, however, are not stained with either AE3 or AE13 (Fig. 10, *c* and *e*).

The AE14 antibody selectively stains the relatively matured cortical cells (Fig. 12 *a*). Compared with AE3 or AE13 staining, this staining starts in a much more advanced region where the thin (2–3 layered) bulbar outer root sheath is replaced by a much thicker (>5–6 layered) outer root sheath (Fig. 12 *a*). This area is equivalent to the upper "elongation" or lower "keratogenous" zone (Orwin, 1979). Since AE14 antibody cross-reacts with the 58K soft keratin (Fig. 2 *d*, lane *1*), it also stains the basal cells of almost all stratified squamous epithelia including the isthmus outer root sheath (Fig. 12, *a* and *c*, and data not shown).

The differential expression of keratins and the AE14-positive antigens in various parts of human hair follicle is summarized in Table I (see Discussion).

Discussion

Defining Subclasses of Human Hair Keratins Using Monoclonal Antibodies

Using AE3, AE12, and AE13 antibodies, we have identified in human hair fibers two keratins, designated 44K and 46K, which belong to the acidic (type I) subfamily, and three keratins, 56K, 59K, and 60K, which belong to the basic (type II) subfamily. In vitro translation experiments have previously suggested that similar keratins in guinea pig, sheep, and mouse probably represent products of distinct genes rather than variants arising from posttranslational processing (Steinert and Rogers, 1971; Ward and Karmarick, 1980; Bertolino et al., 1982). Traditionally called low sulfur keratins, these proteins belong to the intermediate filament family of proteins (Fig. 4 *b*), are highly conserved across mammalian species (Fig. 7), and can always be divided into two mutually exclusive subfamilies according to their size, charge, and im-

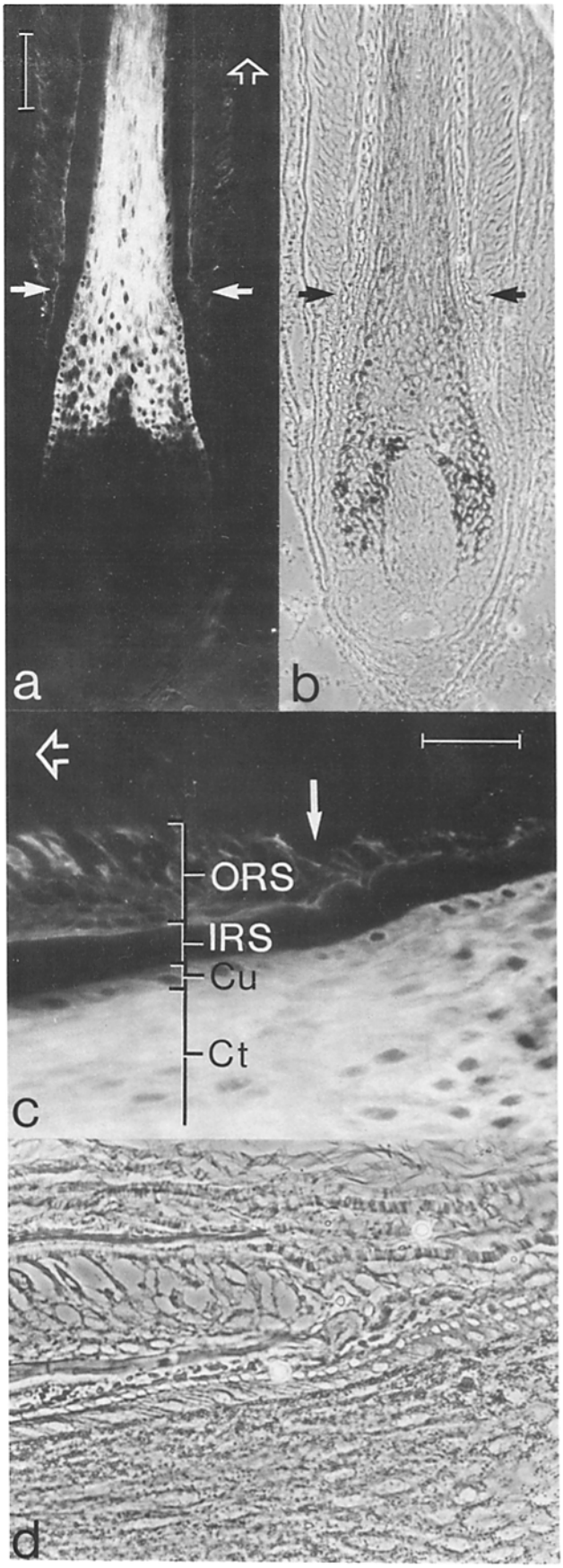

Figure 11. Immunofluorescent staining of a human scalp hair follicle using AE3 mouse monoclonal antibody that recognizes the basic 56–60K low sulfur hair keratin and other basic "soft" keratins. *a* and *c* show AE3 staining, and *b* and *d* are their corresponding phase-contrast images. Symbols are the same as Fig. 9. Note the strong staining of upper cortical and hair cuticle cells, and very weak staining of the entire outer root sheath. Bars, (*a* and *b*) 100 μm; (*c* and *d*) 50 μm.

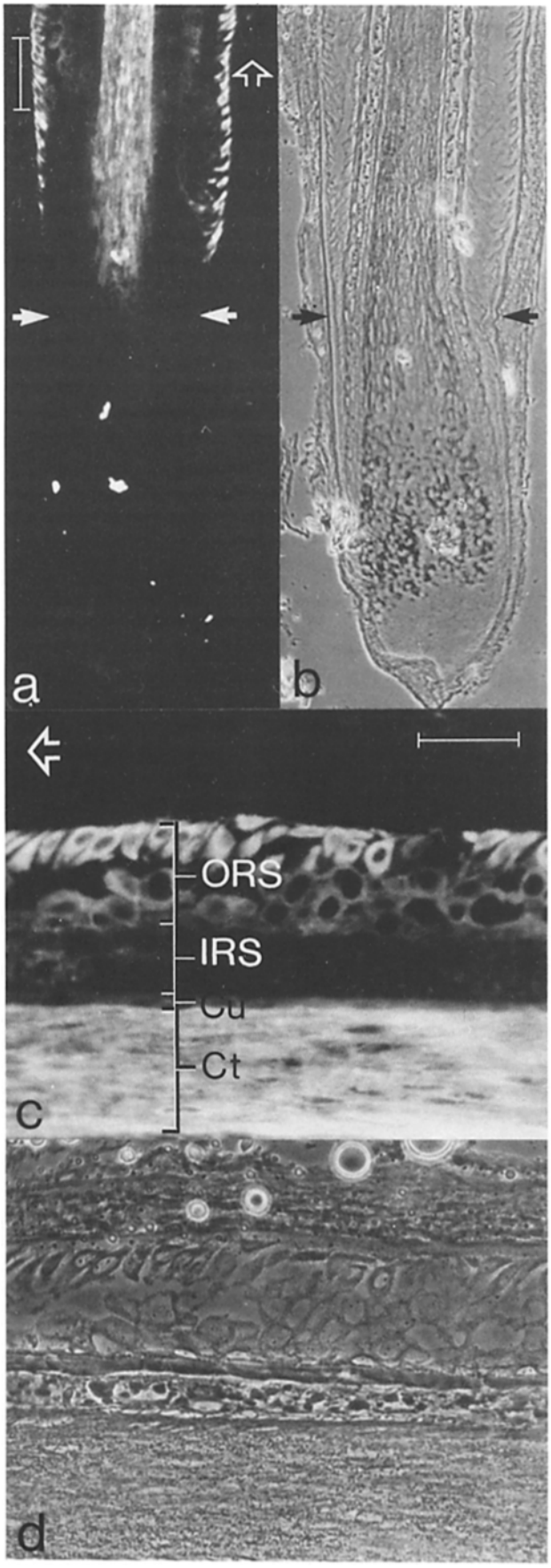

Figure 12. Immunofluorescent staining of a human scalp hair follicle using AE14 mouse monoclonal antibody to the 10–25K, presumptive high sulfur proteins. *a* and *c* show AE14 staining, and *b* and *d* are their corresponding phase-contrast images. Symbols are the same as in Fig. 9. Note the strong staining of advanced (more distal) cortical cells (with no staining in hair cuticle), as well as the preferential staining of the basal cells of isthmus outer root sheath (due to the 58K keratin; see text). Bars, (*a* and *b*) 100 μm; (*c* and *d*) 50 μm.

munoreactivities with our AE13 and AE3 monoclonal antibodies (Figs. 1, 2, 4, and 7; Gillespie and Marshall, 1977; Hewish et al., 1984).

In addition to the low sulfur keratins, human hair fibers also contain a family of 10–25K, AE14-reactive molecules (Fig. 2 *d*, lane 2). Based on their molecular weight and species distribution, we have tentatively identified these as high sulfur proteins. Although historically also known as keratins, they are much smaller than the usual 40–70K size range of intermediate filament subunits, they lack the epitope recognized by aIF antibody (Fig. 1 *c*), and they probably form the interfilament matrix (Gillespie, 1983). Therefore, these proteins may correspond to what is now generally considered as "intermediate filament–associated proteins."

The significance of the common AE14-epitope shared by the 10–25K putative high sulfur proteins and the 58K "soft" keratin (No. 5; which is not present in hair fibers) is currently unclear, and one cannot rule out the possibility that this cross-reaction is purely by chance (Wehland et al., 1984). Nevertheless, the specificity of AE14 antibody makes it useful for the positive identification and localization of the 58K keratin in non-hair/nail epithelia.

The Number of Known Human Keratins

The two-dimensional PAGE patterns of known human keratins including those of the hair and nail are summarized schematically in Fig. 6. The number of known human keratins has therefore reached 23–24 (including 18–19 soft keratins; Moll et al., 1982; Sun et al., 1984). However, this number does not take into account (*a*) keratin isomers resulting from posttranslational modifications (Sun and Green, 1978*a*; Steinert and Idler, 1979; Gilmartin et al., 1984) or minor sequence variations (Tyner et al., 1985; Wild and Mischke, 1986), (*b*) the various keratin-associated proteins such as filaggrin, high sulfur, or high glycine/tyrosine proteins (Dale et al., 1985), or (*c*) the inner root sheath keratins that are potentially distinct but have not yet been isolated in intact form due to a high degree of isopeptide cross-linking (Gillespie, 1983).

Relationship between Soft and Hard Keratins

From the diagram in Fig. 6, it is apparent that, within each of the two keratin subfamilies, the hair keratins are among the most acidic. Based on this subfamily classification and the well-established requirement (in soft keratins) of at least one basic and one acidic subunit for filament assembly (Lee and Baden, 1976; Steinert et al., 1976; Hatzfeld and Franke, 1985; Eichner et al., 1986), it seems almost certain that both acidic and basic hair keratins will be required for filament formation (cf., Ahmadi and Speakman, 1978; Gruen and Woods, 1983).

Within the category of soft keratins, most acidic keratins form pairs with specific basic keratins as defined by (*a*) fre-

Table I. Differential Expression of Keratins in Human Hair Follicles

Cell type	44/46K hard-acidic (AE13)	56-60K hard-basic (AE3)	10-25K high sulfur (AE14)	Soft keratins (AE1, AE3)
Bulbar matrix	−	−	−	−
Upper cortex	+	+	+	−
Upper hair cuticle	+	+	−	−
Inner root sheath	−	−	−	−
Outer root sheath	−	−	−	+

Parentheses denote antibody reactivities.

quent coexpression (Sun et al., 1984), (*b*) shared epitopes as recognized by monoclonal antibodies (e.g., 56.5K and 65–67K keratins share the epitope recognized by AE2, and 45K and 52K keratins share the epitope recognized by 6.11 antibody; reviewed in Cooper et al., 1985), and (*c*) preferential in vitro interaction (Franke et al., 1983; Eichner et al., 1986). Our immunolocalization and tissue distribution data strongly suggest that the acidic 44K/46K and basic 56–60K hair keratins are co-expressed and thus may be regarded as another "keratin-pair" that may serve as a marker for "hair/nail-type" epithelial differentiation (Table II).

The question arises why are there multiple acidic and basic members within the hair keratin "pair." Actually, the existence of a keratin "pair" comprised of more than two polypeptides is not without precedent. For example, in the "skin-type" differentiation pair, the two basic polypeptides, 65K and 67K, are matched with the 56.5K acidic polypeptide (Table II). As keratin pairs are defined by the specific co-expression of both the acidic and basic members in a differentiation-dependent manner, co-regulated polypeptides within the same subfamily may be regarded, at least conceptually, as one member of a pair. Whether the 44K and 46K hair components (or the 56K/59K/60K component) are functionally identical or different remains to be determined.

An interesting feature of keratin pairs is that in most cases the basic member is larger than the acidic one by 8–10K (Sun et al., 1984). Recent DNA sequencing data suggest that this mass difference is due to the presence of specific "H1" and "H2" domains bracketing the central alpha-helical segment of the basic keratins (Steinert et al., 1985). Whether a similar situation exists for the hair keratins and why the basic and acidic members of the hair pair differ by 12–14K instead of the usual 8–10K is unclear.

Differential Expression of Hair Proteins

The fact that AE3 (recognizing the basic, type II, hair keratins) and AE13 (specific for the acidic, type I, hair keratins) stain identically the upper cell layers of hair matrix (Figs. 10 and 11) suggests that the basic and acidic hair keratins appear coordinately during advanced stages of hair differentiation. Although the absence of hair-specific keratins in matrix basal cells will have to be confirmed eventually by direct biochemical analysis, our finding is consistent with the morphological observation that lower bulbar cells contain relatively few desmosome-associated, 10-nm filaments (Birbeck and Mercer, 1957; Roth and Helwig, 1964; Hashimoto and Shibazaki, 1976). The suprabasal location of hair-specific keratins is also in keeping with our current concept that most, and probably all, of the larger molecular weight, differentiation-specific (soft) keratin pairs are suprabasally expressed (Table II). These include the 56.5K/65–67K keratin markers for "skin-type" differentiation (Woodcock-Mitchell et al., 1982), the 55K/64K keratin markers for "corneal-type" differentiation (Schermer et al., 1986), and the 51K/59K keratins markers for "esophageal-type" differentiation (Schermer, A., and T.-T. Sun, unpublished data; Table II). If the hair keratin pair indeed falls into this category, we can predict that its expression will be differentiation dependent; i.e., its synthesis will be modulated greatly depending on the growth and differentiated state of the matrix cells (for a discussion see Cooper et al., 1985).

The 10–25K high-sulfur proteins, as defined by our AE14 antibody, are first detected in cortex ~20–30 cell layers above the basal cells (Fig. 12). A related finding is that intermediate filament bundles isolated by Jones and Pope (1985) from the lower bulb of human hair follicles do not contain high sulfur proteins. Although this finding alone does not prove that high sulfur proteins are absent from the lower hair

Table II. Keratin Markers for Different Types of Human Epithelial Differentiation

Markers	Keratins: Catalogue* (Basic/Acidic)	Keratins: Molecular weight*‡ (Basic/Acidic)	Tissue distribution
Skin-type differentiation§	1,2/10	65-67/56.5	Keratinized (cornified) stratified squamous epithelia
Corneal-type differentiation§	3/12	64/55	Central corneal and adjacent limbal epithelia
Esophageal-type differentiation§	4/13	59/51	Most nonkeratinized (noncorneal) stratified squamous epithelia
Palm/Sole-type differentiation§‡‡	(1,2)/9	(65-67)/63	Thick, glabrous epithelia of palm and sole
Hair/Nail-type differentiation§	−	56,59,60/44,46	Hair shaft and nail plate
Keratinocytes‖	5/14,15	58/50,50′	Keratinocytes and related cells
Hyperproliferative keratinocytes¶	6/16,17	56/48,46	Embryonic, cultured, and certain diseased keratinocytes (including carcinoma)
Simple epithelia	8/18	52/45**	Mainly simple epithelia and related cells

* Basic (type II) keratins are underlined.
‡ Molecular weight × 10^{-3}. Note the differences of ~8,000 between the basic and acidic members of most keratin pairs, and the exceptionally large size difference of 12,000–14,000 of the hair "pair."
§ Represent differentiation-specific keratins that are mainly expressed in suprabasal cell layers.
‖ Represent the two major keratins of basal keratinocytes.
¶ Almost always expressed in conjunction with the 5/14 pair and may co-exist with differentiation-specific keratins.
** The No. 7 (54K) and No. 19 (40K) keratins are mainly found in simple epithelia; they do not seem to have regularly coexpressed "partners" and are, for simplicity, not included in this table.
‡‡ Knapp et al., 1986.

bulb, since they could have been lost during the isolation procedure, when taken together with our immunofluorescent staining data (Figs. 10–12) it supports the notion that the synthesis of the low sulfur keratins precedes that of the high sulfur proteins (for an opposing view, see Fraser, 1969). These results are also consistent with the earlier suggestion that high-sulfur proteins may function as a matrix only during advanced stages of hair formation (Fraser et al., 1972). In this regard, it would be interesting to see whether high sulfur proteins of the hair can induce the aggregation of in vitro–assembled hair, nail, or epidermal keratin filaments (Dale et al., 1985).

Our immunohistochemical staining data also yield information on the keratin composition of the inner root sheath and the hair cuticle. Since no AE3 or AE13 staining was observed in inner root sheath (Figs. 10 and 11), our immunological data support the current belief that keratin filaments of inner root sheath are distinct from those of the cortex (Steinert et al., 1971; Steinert, 1978).

In cuticle cells, x-ray diffraction and electron microscopy have failed to reveal any alpha-helical, fibrous elements (Birkbeck, 1964), and it has been stated that wool and kangaroo hair cuticles do not contain any low sulfur keratins (e.g., Crewther, 1976). But the fact that human cuticle cells are stained by our AE13 and AE3 antibodies (Figs. 10 *e* and 11 *c*) strongly suggests that these cells contain keratin-related antigens. On the other hand, since cuticle cells are AE14 negative, they probably do not contain the 10–25K high sulfur proteins (Fig. 12 *c*). This finding, of course, does not exclude the possibility that the cuticle cells, which are known to be cystine-rich (Lindley, 1977; Spearman, 1977 *a*, *b*), may express some other forms of high sulfur components (Crewther, 1976; Frater, 1976). In support of this possibility, Ley and Crewther (1980) have actually isolated and characterized some so-called ultra-high-sulfur proteins that appear to be enriched in the cuticle.

Nail Plate Contains Hair Keratins in Addition to the 50K/58K and 48K/56K Soft Keratins

It has been reported that keratins of human nail are almost identical to those of the hair (Baden et al., 1973; Marshall, 1983; cf. Marshall and Gillespie, 1977), and that nail keratins do not cross-react with antisera to epidermal keratins (Lee et al., 1976; Baden and Kubilus, 1983, 1984; cf., O'Guin et al., 1986). However, our data indicate that nail plate contains, in addition to an apparently complete set of hair keratins, ~10–20% of soft keratins. Furthermore, immunoblotting data have allowed us to identify these "soft" keratins as the 50K/58K keratin pair which we have previously identified in the basal cells of several stratified squamous epithelia (Woodcock-Mitchell et al., 1982; Schermer et al., 1986), and the 48K/56K keratin pair which is characteristic of most "hyperproliferative" keratinocytes (Weiss et al., 1984). The presence of the 50K/58K and 48K/56K keratin pairs in nail plate is entirely consistent with nail matrix being an unusually thick, and therefore probably highly proliferative, stratified squamous epithelium (Zaias and Alvarez, 1968). The "non-keratinized" morphology (i.e., lacking a well-defined granular layer) of nail matrix is also consistent with the absence of the 56.5K/65–67K keratin markers of "keratinization" or, as we have proposed recently as an alternative and perhaps less confusing term, "skin-type differentiation" (Cooper et al., 1985; Cooper and Sun, 1986).

The fact that nail plate and mature hair share a similar set of major hard keratins (Figs. 1, 2, and 5) supports the notion that the precursor cells (matrix) that give rise to the nail plate and hair cortex share a common pathway of terminal differentiation (cf., Baden et al., 1973).

Concluding Remarks

Studies on hair follicle differentiation have been hindered by the extreme complexity of hair proteins. However, through the use of monoclonal antibodies we have been able to distinguish immunologically several classes of hair-specific markers, and to correlate them with the classically defined stages of histological differentiation. A detailed analysis of the expression of these markers should lead to a better understanding of the mechanisms of hair development, growth and disorder.

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Note Added in Proof. Subsequent to the acceptance of this manuscript for publication, Heid, H. W., E. Werner, and W. W. Franke (*Differentiation*, in press) have published similar data on the keratin composition of human hair roots, and provided evidence that all of the human hard keratins, similar to those described here, are distinct translational products [4 acidic (Hal-4) and 4 basic (Hbl-4) components].

Two additional papers have appeared (Ito, M., T. Tazawa, N. Shimizu, K. Ito, K. Katsuumi, Y. Sato, and K. Hashimoto, 1986, *J. Invest. Dermatol.*, 86:563–569; and French, P. W., and D. R. Hewish, 1986, *J. Cell Biol.*, 102:1412–1418) describing the preparation of monoclonal antibodies to hair and wool keratins, respectively.

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