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Epidermolytic Hyperkeratosis: Ultrastructure and Biochemistry of Skin and Amniotic Fluid Cells from Two Affected Fetuses and a Newborn Infant*

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Skin biopsy samples and amniotic fluid cells obtained in utero from two fetuses at risk for epidermolytic hyperkeratosis were examined by light and electron microscopy. Both fetuses were affected; the second was carried to term. Epidermal extracts were prepared from blisters of the newborn for analysis of keratin and filaggrin proteins. Abnormal clumps of keratin filaments were present in all layers of the prekeratinized fetal epidermis except the periderm and stratum germinativum. A significant population of amniotic fluid cells also contained the filament aggregations. Prenatal diagnosis of the disease should be possible using cells obtained at amniocentesis, thus avoiding fetal skin biopsy. Biochemical studies showed abnormalities in keratin and filaggrin proteins. The structural alterations in the tissue might be a consequence of altered interaction between these two abnormal epidermal proteins.

Epidermolytic hyperkeratosis (congenital bullous ichthyosiform erythroderma) is an autosomal dominant disorder of keratinization that is characterized histopathologically by hyperkeratosis, intraepidermal bullae, and by the presence of condensed keratin (tonofilament) bundles in spinous and granular cells [1–5]. The filamentous accumulations serve as the primary morphologic markers of the disease in the tissue. The mitotic rate of epidermal cells is increased severalfold over normal; correspondingly, the transit time is decreased [6]. A biochemical basis for the disease has been suggested by the finding of decreased amounts of fibrous proteins in the epidermis and the absence of 1 of the keratin polypeptides [7].

The clinical presentation of the disease is an initial erythroderma and blistering. The erythroderma usually fades, blistering improves, and hyperkeratosis develops. Intense buildup of scale is particularly prominent in flexural creases and other intertriginous areas [2]. Improvement is usually seen with age [1].

The disorder has been diagnosed in utero in at least 3 separate instances [8,9] on the basis of the ultrastructural identification of filament aggregations in fetal skin biopsy specimens obtained at 19–20 weeks gestation.

We have examined by light and electron microscopy the skin biopsy samples and amniotic fluid (AF) cells from 2 fetuses of the same family who were at risk for epidermolytic hyperkeratosis. Structural data from skin samples from 1 of these fetuses have been reported [8]. Biochemical studies of proteins in blister epidermis and cornified scale obtained from the affected infant (born from the second pregnancy) were also carried out. The objectives of these studies were: (1) to document more fully any structural characteristics of prekeratinized fetal epi-

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Abbreviations:
AF: amniotic fluid
SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TEM: transmission electron microscopy

dermis that may be diagnostic for the disorder, (2) to determine whether any cells of the AF identified by light or transmission electron microscopy reflect the abnormal condition in the skin, hence may suggest amniocentesis as an alternative or supportive method of prenatal diagnosis, and (3) to identify biochemical alterations in epidermal proteins of keratinization that could provide a molecular basis for the observed structural abnormalities.

MATERIALS AND METHODS

Light and Electron Microscopy

Two fetuses from the same family who were at risk for epidermolytic hyperkeratosis were biopsied in utero at 19 weeks gestational age for prenatal diagnosis of the disorder on the basis of structural abnormalities in the skin. AF samples were collected from both fetuses prior to the biopsy procedures. The procedures of fetoscopy and fetal biopsy and the assurance of genetic counseling of the parents and their advised consent for the procedures have been described elsewhere [8]. The first fetus was diagnosed abnormal by the finding of condensed filament aggregations and vacuolate cytoplasm in suprabasal epidermal cells and was subsequently aborted [8]. The second fetus was also biopsied in utero but was carried to term and at birth found to be a male of normal birth weight affected with the disease.

The skin biopsy specimens were placed immediately into minimum essential medium supplemented with 20% fetal calf serum, examined, and then immersed in 1.5% glutaraldehyde in 1.2 M cacodylate buffer. AFs were diluted with the same fixative and centrifuged to form a pellet of the cells. All samples were postfixed in 1% OsO_4 in distilled water for an additional hour, dehydrated, and embedded in Araldite. One-micron sections were cut for light microscopy and stained by the method of Richardson, Garrett, and Finke [10]. Thin sections (80 nm) were prepared for transmission electron microscopy (TEM), stained with warm alcoholic uranyl acetate and lead citrate [11], and examined in a Philips 201 transmission electron microscope.

Biochemistry

Extraction and separation of epidermal proteins: Squames collected from hyperkeratotic epidermis and partial-thickness sheets of blister roof epidermis obtained from the newborn infant were extracted for biochemical studies of keratin proteins and filaggrin (also known as epidermal matrix protein, histidine-rich protein, or stratum corneum basic protein). The epidermis was homogenized in 8 M urea containing 0.1 M Tris-HCl, pH 7.5, 0.1 M 2-mercaptoethanol, 1 mM dithiothreitol, and 20 g/ml phenylmethylsulfonyl fluoride. Samples were stirred 2–4 h and then centrifuged at 20,000 *g* to remove the insoluble material.

Extracted proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using the method of Laemmli [12]. Prior to electrophoresis, protein samples were incubated at 100°C for 2–5 min in buffer containing 1.5% SDS and 1.5% 2-mercaptoethanol. Gels were stained with Coomassie Brilliant Blue, destained, and photographed.

Immunologic detection of antigen (filaggrin): Detection of filaggrin on SDS gels was done by a modification of the method of Renart, Reiser, and Stark [13]. Protein bands were transferred electrophoretically to diazobenzyloxymethyl (DBM)-paper in 50 mM sodium phosphate buffer, pH 6.5. The DBM-paper was prepared from aminobenzyloxymethyl (ABM -paper [Trans-bind], Schleicher & Schuel, Inc.) according to the manufacturer's instructions. The paper containing the transferred proteins was incubated with antiserum to rat epidermal filaggrin or preimmune control serum, each diluted 1:50 in buffer I [13]. After washing with 2 changes of buffer I, the paper was incubated with ^{125}I-protein A (approximately 0.25 μCi/gel slot) for 2 h, washed, and dried. Autoradiographs were made using Kodak X Omat film.

RESULTS

Ultrastructural Characteristics of the Epidermis of a Normal 20-Week Gestation Fetus

The interfollicular epidermis consists of one basal layer, 2–4 intermediate cell layers, and a superficial layer of periderm (Fig 1). The basal and intermediate cells contain prominent bundles of keratin filaments (tonofilaments) and a significant quantity of glycogen. The uppermost intermediate cells are flattened and have a greater condensation of filaments, suggesting the onset of keratinization. The periderm is "regressed" and characterized morphologically by a filamentous cytoplasm, few surface microvilli, flattened blebs, and a cornified cell envelope [14]. The epidermis is keratinized only in portions of developing hair follicles. The superficial portion of the follicle (hair canal) penetrates the epidermis at this stage of development and forms a diagonal tract of keratinized cells within the epidermis. Granular cells surround these channels concentrically but do not appear in the interfollicular epidermis until 22–24 weeks gestation [15].

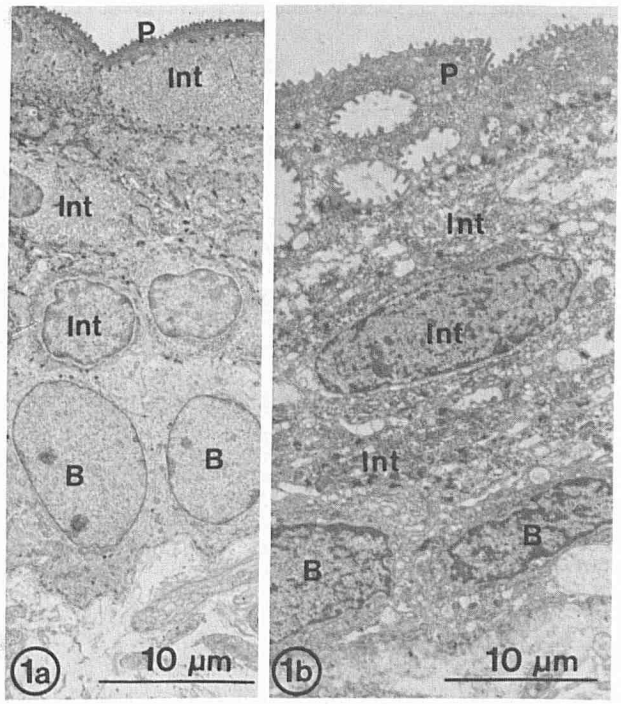

FIG 1. Epidermis from normal 19- to 20-week-old human fetuses sampled postmortem (*a*) or by in utero biopsy (*b*). The tissue obtained by fetal biopsy is vacuolated and less well preserved by comparison with the other specimen. Periderm (*P*), intermediate (*Int*), and basal (*B*) cell layers are shown in both specimens.

Ultrastructural Characteristics of the Epidermis from the Two Fetuses at Risk

Skin biopsy specimens (Fig 2) from the 2 fetuses at risk were identical to controls in that: (1) the thickness, organization, and number of epidermal cell layers was normal, (2) a granular layer was not yet apparent and the interfollicular epidermis was not keratinized, (3) keratinization was apparent in hair follicles and hair canals, but hairs were not exposed at the surface, and (4) a regressed periderm covered the surface in all areas except at sites where hair canals were eroded open at the epidermal surface. In contrast with the normal tissue, the cytoplasm of epidermal cells from both fetal biopsies was poorly preserved and all cells except those of basal and periderm layers contained highly condensed bundles of tonofilaments. These were irregular in shape and size and attached to the intracellular plaques of the desmosomes (Figs 2,3). The quantity of filaments involved in the aggregates varied among cells regardless of the level of the layer. In some cells, a normal distribution of filaments was observed in addition to the condensed bundles (Fig 3*a*). The morphology of granular and cornified cells surrounding the hair canals was examined to evaluate the state of filament aggregation in cells that are more differentiated than intermediate cells (Fig 4). In the granular cells, small keratohyaline granules were recognized, independent of the condensed filament bundles (Fig 4*a*). This is in contrast to the filament bundle–keratohyalin associated aggregates described in the ep-

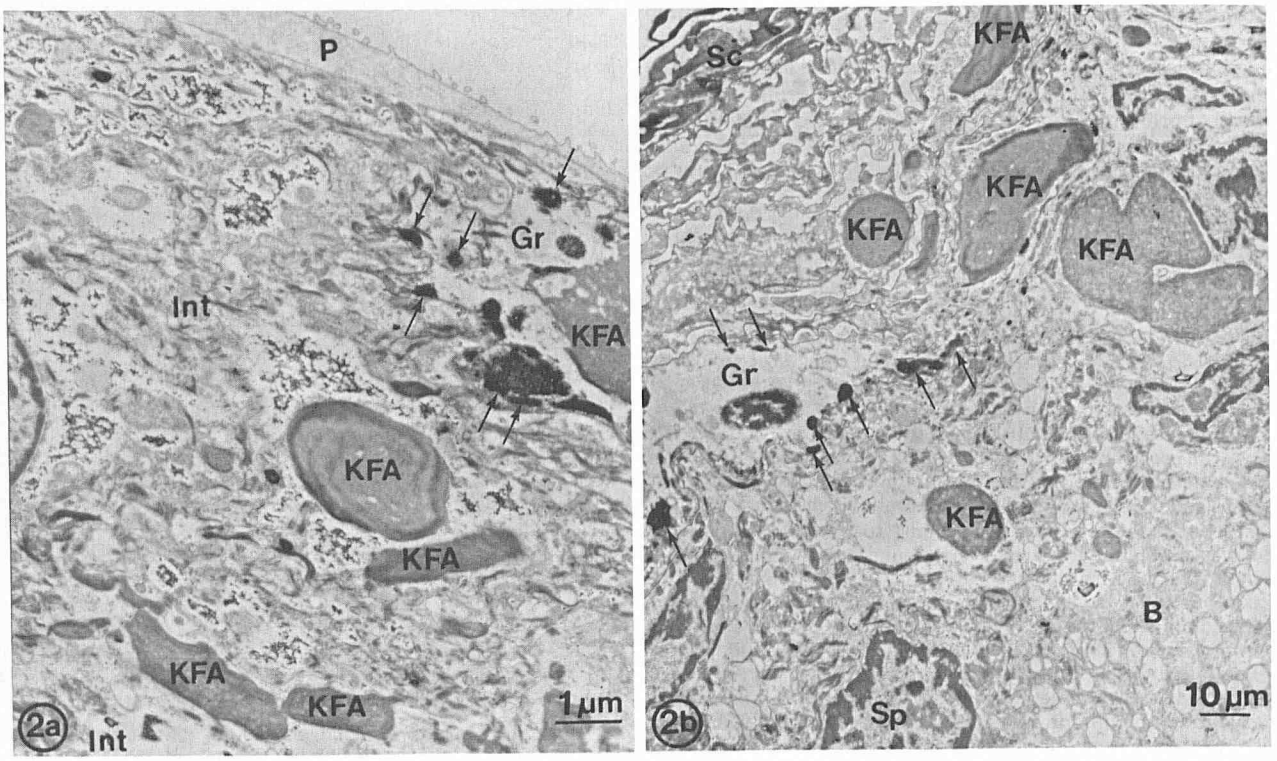

FIG 2. Epidermis of the interfollicular (*a*) and follicular (*b*) regions of the skin from a 19-week-old fetus affected with epidermolytic hyperkeratosis. Keratin filament aggregations (*KFA*) are evident in granular (*Gr*), spinous (*Sp*), and intermediate (*Int*) cells. Periderm (*P*), cornified (*Sc*), and basal (*B*) layers are also evident. The granular cells that border a hair canal (*a* and *b*) contain keratohyaline granules (*arrows*).

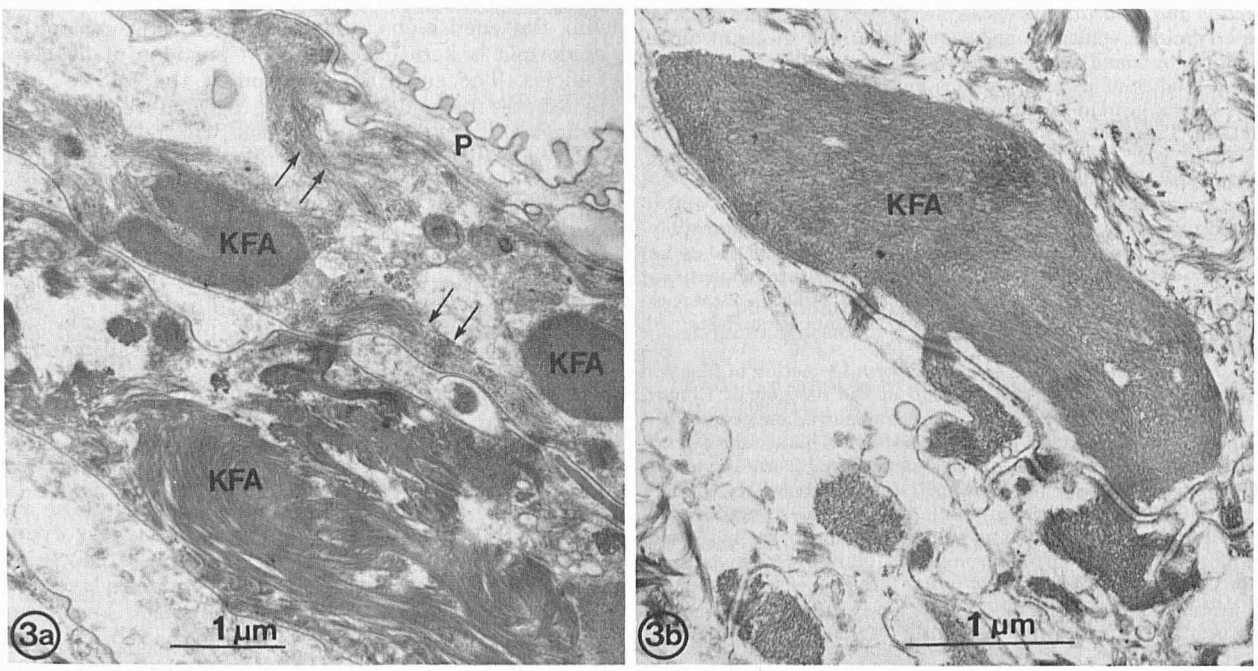

FIG 3. Keratin filaments in intermediate cells condensed to various degrees. Aggregated (*KFA*) and dispersed (*arrows*) filaments are evident (*a*). The aggregations may be associated with desmosomes (*b*). Periderm (*P*) and intermediate cells are shown.

idermis of adult patients with the disorder [4,16]. All of the cornified cells of the hair canals had a finely reticulate or "mottled" pattern (Fig 4*b*) which would be described as abnormal in adult skin but is typical of the first keratinized cells seen in utero in normal fetuses in both follicular and interfollicular epidermal regions; it is a pattern of keratinization which has been speculated to be "incomplete," to occur without full participation of the differentiated forms of matrix and filamentous proteins [17]. Remnants of organelles were often seen in these squames (Fig 4*b*).

Histology and Ultrastructure of the AF Cells

Four different AF cell pellets from the 2 fetuses were examined by light and electron microscopy (Figs 5,6). The majority

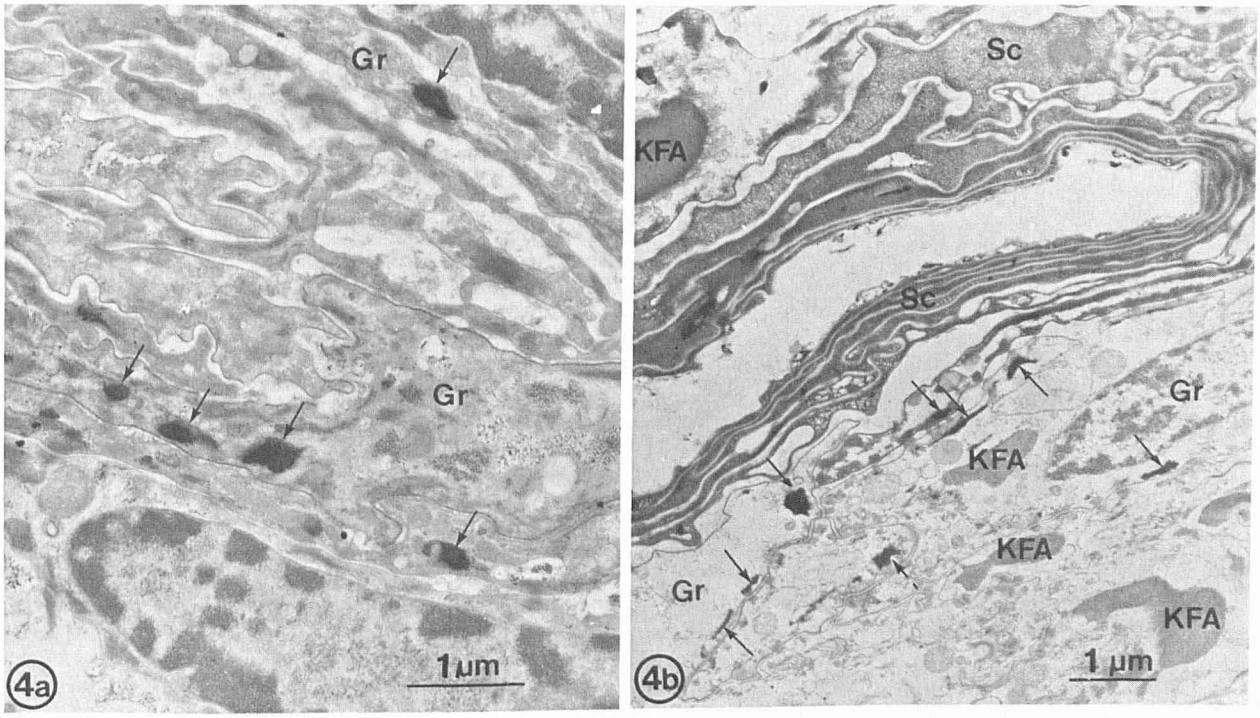

FIG 4. Cornified (*Sc*) and granular (*Gr*) cells surrounding a hair canal. Keratohyaline granules (*arrows*) are not associated with keratin filament aggregations (*KFA*).

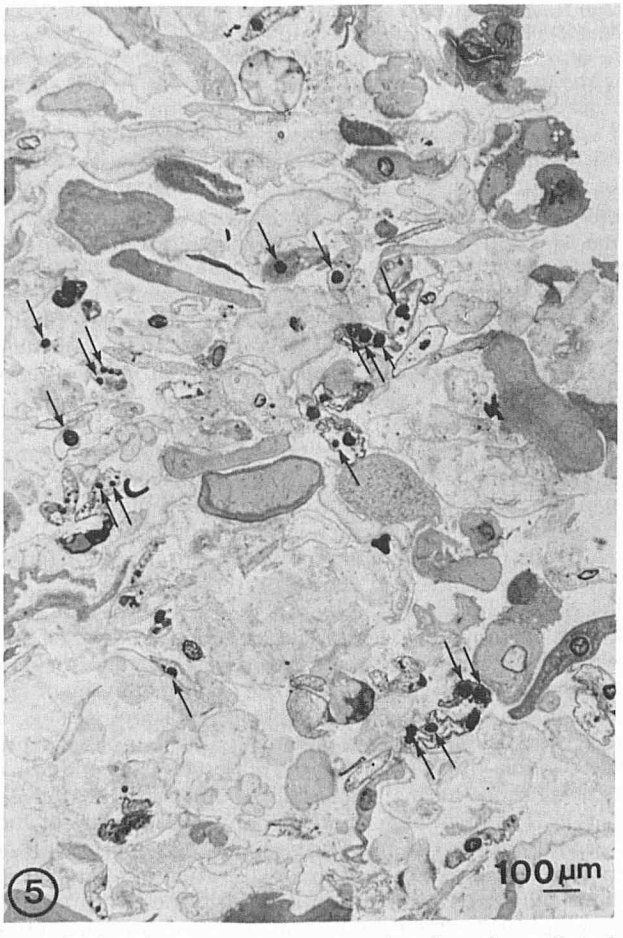

FIG 5. Light micrograph of a 1-µm section through a pellet of AF cells obtained in utero from the affected fetus. Keratin filament aggregations (*arrows*) are evident in several of the cells.

of cells were recognized as periderm at the light microscopic level by either shape, abundance of glycogen, sharply defined cell outline, or pale cytoplasm (Fig 5). The differences in periderm morphology relate to their stage of differentiation [14]. Macrophagic cells, cells of the amnion, and occasional chunks of tissue (evidently epidermal in origin) also were present. Most notable, however, was a significant population of cells which contained large, darkly stained granules and a pyknotic nucleus (Fig 5). We presumed that these cells were derived from the epidermis and that the darkly staining granules were the condensed filament aggregates. Thin sections of all AF pellets examined by TEM confirmed the identity of these cells (Fig 6). The "granules" were readily identified as condensed bundles of filaments identical to those present in the epidermis of affected fetuses. All sections from each AF cell pellet contained a large enough quantity of these cells to suggest that the diagnosis of epidermolytic hyperkeratosis might be made on the basis of finding these cells in AF samples obtained by amniocentesis.

Biochemistry of Epidermal Extracts From the Affected Newborn and His Mother

Proteins extracted from blister epidermis (Fig 7, lane 3) and cornified scales (lane 4) of the newborn were separated by SDS-PAGE. These were compared with a similar extract derived from a normal newborn (lane 2). The proteins that were of primary interest were the keratins, the main structural proteins of epidermis, and filaggrin, the presumed keratin matrix protein [18].

The keratins were identified as darkly stained bands between M_r 46,000 and 68,000. In the extract of normal epidermis (Fig 7, lane 2), 2 main groups of keratins had M_rs of 67,000 and 64,000, and 56,000, 54,000, and 52,000, respectively. In extracts of the blister epidermis (lane 3) and scale of the affected infant (lane 4), the keratin bands were less distinct and faster migrating than those in the extract of normal epidermis, suggestive of proteolytic damage. In the affected infant epidermis, the M_r 64,000 and 67,000 keratins were replaced by 62,000 and 60,000 bands.

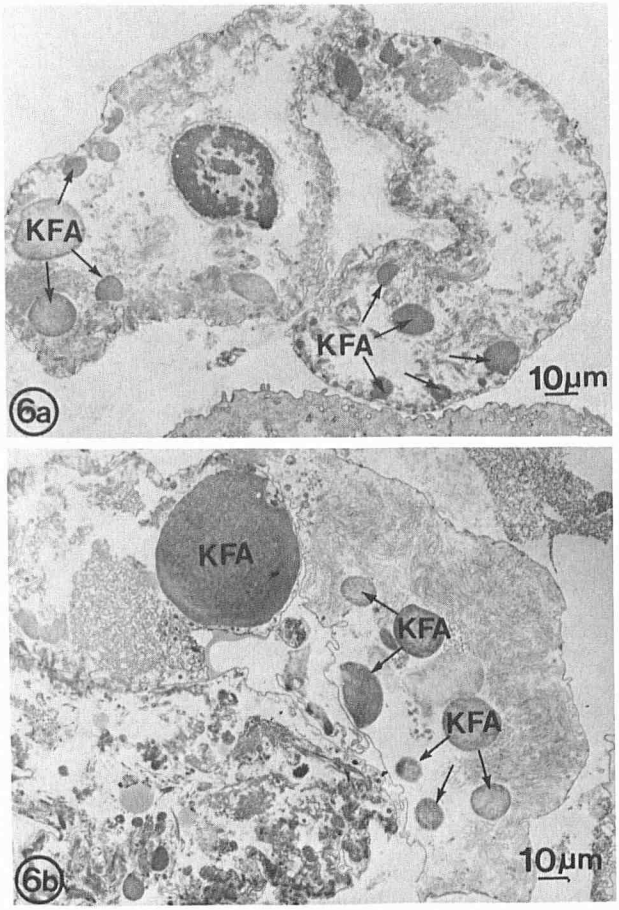

FIG 6. *a* and *b*, Electron micrographs of two amniotic fluid cells containing keratin filament aggregations (*KFA*).

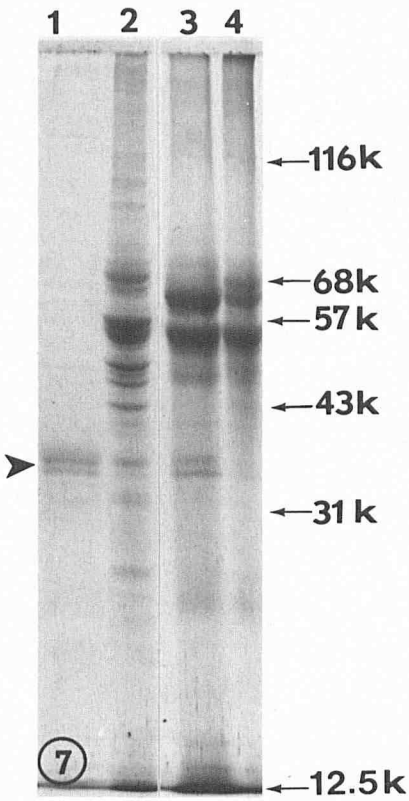

FIG 7. SDS-PAGE of proteins extracted from the epidermis of a normal newborn (*lane 2*), and the epidermal blister (*lane 3*) and scale (*lane 4*) from the affected newborn infant. Filaggrin (*arrow*) is shown as a doublet of approximately 35K M_r. Keratin filament proteins band in the M_r range of 52–67K. The numbers on the gel were determined from M_r standards.

Filaggrin was identified in the normal as a cationic protein doublet of approximately 35,000 M_r (Fig. 7, lane 1) which cross-reacts with antiserum to rat filaggrin (data not shown). This doublet can be identified in the extract of normal epidermis (lane 2) and is quite prominent in that of the affected infant. It is absent in scale extracts. Preliminary immunoautoradiographic studies suggest that filaggrin cross-reactive protein in the affected infant migrated at a position of approximately 35,000 M_r, corresponding with filaggrin in extracts of normal epidermis. Some immunoautoradiographs also showed cross-reactive proteins at lower (~15,000) and higher (~70,000) M_rs in addition to that at 35,000. Filaggrin in the extract of the affected infant epidermis showed a significantly greater reactivity with the antibody than any other human sample tested by this procedure.

DISCUSSION

Condensed aggregations of keratin filaments were identified ultrastructurally in the epidermis and AF cells obtained in utero from 2 fetuses at risk for epidermolytic hyperkeratosis. These structures were identical to the primary alteration in epidermal cells of affected adults and were the only structural abnormality observed in the fetal skin biopsies when compared with those from normal age-matched fetuses. In contrast to the original observation of the epidermis from the first of the affected fetuses [8], keratohyaline granules associated with hair canals and follicles were normal in structure, and not globular. The preservation of the tissue was not sufficient to distingush pathologic vacuolization of the cytoplasm (as reported) from preparation artifact. There was no evidence of early-onset keratinization or hyperplasia of the interfollicular epidermis of the hair follicle or canal as described for affected adult epidermis [1–5]. It is possible, however, that additional structural abnormalities and changes in kinetic properties characteristic of the disease postnatally, may be expressed in utero after keratinization, and once a rate of epidermal turnover equivalent to the newborn, is established.

The observation of altered filament organization suggests abnormalities in either the keratin filament and/or filament-associated (filaggrin) proteins. Biochemical studies of the epidermis from the affected newborn showed that the high M_r keratins (67,000 and 64,000) were absent and replaced by proteins of M_r 62,000 and 60,000. Ogawa et al [7] have also reported abnormalities in keratins from the epidermis of a patient with epidermolytic hyperkeratosis, but in contrast with our studies, they found that a low M_r keratin (55,000) was absent. We have also observed an apparent increase in filaggrin at M_r 35,000 and extra bands of filaggrin immunoreactive protein not found in normal human epidermal extracts.

It has been hypothesized by others [7], that the exaggerated cohesion of keratin filaments is a consequence of abnormalities in the keratin polypeptides. We propose that abnormalities in filaggrin might also contribute to the abnormal filament aggregation. It has been shown that filaggrin can promote aggregation of keratin filaments in vitro [18] and that the structures formed mimic the keratin pattern in tissue. The conditions for producing such "normal appearing" aggregations are quite specific [19–21] and an abnormality in any one of them, including an abnormal filaggrin protein or an altered concentration of a normal filaggrin, might lead to an alteration in filament aggregation. It has also been shown that histidine-rich proteins are present in cells of all epidermal layers of human fetal skin before 4 months gestation. They concentrate in upper intermediate cell layers after that time [22]. Similar observations

have been made in fetal rat epidermis [23]. However, skin from individuals affected with epidermolytic hyperkeratosis from other families will need to be examined for alterations in filaggrin before this protein can be implicated in the molecular mechanism of the disease.

Abnormalities in keratins have been demonstrated in other diseases of keratinization [24–27]; abnormalities in histidine-rich proteins (presumably filaggrin) have been associated with certain hyperproliferative diseases [28,29]. A block in the conversion of filaggrin precursor to filaggrin is presumably related to the absence of terminal differentiation in a genetic animal model of abnormal keratinization [30]. The present study may be the first instance where an excess of filaggrin in the epidermis of an individual with a disease of keratinization has been reported.

The finding of sheets of cells and individual cells in the AF which contain keratin filament aggregations is significant, with respect to prenatal diagnosis. We suspect that these cells are released into the AF as a consequence of the formation and rupture of epidermal blisters. Normal AFs contain only 25% of epithelial cells and these are presumed to be derived primarily from gastrointestinal and respiratory epithelia [31]. The cells we have observed are clearly epidermal in origin. Their presence suggests that prenatal diagnosis of epidermolytic hyperkeratosis in utero may be possible using samples of AF. The use of AF for diagnosis might eliminate the need for the higher-risk procedures of fetoscopy and fetal biopsy, and at least, would provide a second source of material to search for the disorder, thereby alleviating the problems inherent in basing diagnosis on a small skin biopsy sample. Amniocentesis is performed earlier than fetal biopsy (14–16 weeks gestation), therefore the diagnosis might be made earlier in fetal life and without requiring the mother to travel to one of the few fetoscopy centers in this country. There has been, however, no experience in examining skin from fetuses affected with the disorder at ages younger than 19 weeks, thus it is not known how early the abnormal cells appear in the AF. The timing of appearance must depend upon the synthesis of keratin and filaggrin proteins in sufficient quantities to form aggregates. Detection of the abnormality in AF cells would appear to require blister formation to interrupt the integrity of the epidermis. At what age this begins in utero is unknown. Biochemical studies of normal fetal skin at 14–16 weeks gestation age can answer the question of the appearance and quantity of the epidermal proteins, but the question of disease expression at this age requires the identification of a fetus at risk and a family that is anxious for the disorder to be recognized prenatally.

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