A Novel Dinucleotide Mutation in Keratin 10 in the Annular Epidermolytic Ichthyosis Variant of Bullous Congenital Ichthyosiform Erythroderma

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Annular epidermolytic ichthyosis has recently been delineated as a distinct clinical phenotype within the spectrum of epidermolytic keratinization disorders. The pattern of inheritance of the disorder is consistent with an autosomal dominant mode of transmission. Here we report a second incidence of this disorder in a family with two affected generations. The proband suffered from bullous ichthyosis and had bouts of disease activity associated with the development of numerous annular and polycyclic erythematous, hyperkeratotic plaques on the trunk and the proximal extremities. Histologic examination showed the typical pathology of epidermolytic hyperkeratosis, and ultrastructural analysis revealed abnormal keratin filament networks and tonofilament clumping with a perinuclear distribution. Molecular analysis revealed a novel tandem CG to GA 2-bp mutation in the same allele of keratin 10 in affected individuals, resulting in an arginine to glutamate substitution at residue 83 (R83E) of the 2B helical segment. We conclude that annular epidermolytic ichthyosis should be considered a variant of bullous congenital ichthyosiform erythroderma. ***Key words: intermediate filaments/disease/genetics. J Invest Dermatol 108:357–361, 1997***

The term epidermolytic hyperkeratosis has gained widespread usage to describe both a distinct clinical entity, bullous congenital ichthyosiform erythroderma (BCIE) (Frost *et al*, 1966), as well as the histopathology associated with the various types of bullous ichthyoses and certain types of palmoplantar keratoderma (Hamm *et al*, 1988; Traupe, 1989). This histopathology is characterized by vacuolar degeneration of keratinocytes, blurred cell boundaries, and the presence of abnormal keratohyalin granules (Ackerman, 1970). Electron microscopy of these disorders invariably reveals abnormal keratin filaments with tonofilament clumping and a collapsed keratin filament cytoskeleton often with a perinuclear distribution (Anton-Lamprecht, 1994). The use of the same term to describe both a histopathology and a distinct disorder has caused some confusion because it is now evident that a number of different genetic keratinization disorders exhibit similar pathologic changes, and many of these have recently been shown to involve keratin mutations (Rothnagel, 1996). For example, BCIE is caused by mutations in either keratin 1 or keratin 10 (Cheng *et al*, 1992; Chipev *et al*, 1992; Rothnagel *et al*, 1992) which are preferentially expressed in the lower spinous layer of the epidermis. In contrast, mutations in keratin 2e, which is expressed in the upper spinous layer, are associated with ichthyosis bullosa of Siemens (IBS) (Kremer *et al*, 1994; McLean *et al*, 1994; Rothnagel *et al*, 1994). The differential expression of these keratins correlates with the clinicohistologic observation that IBS runs a much milder course than BCIE and that in IBS, the histopathology of epidermolytic hyperkeratosis is confined to the uppermost layers of the stratum spinosum and the granular layer (Traupe *et al*, 1994). In epidermolytic palmoplantar keratoderma, mutations in keratin 9 have been found consistent with the restricted expression of this keratin to the epidermis lining the palms and soles (Bonifas *et al*, 1994; Reis *et al*, 1994; Rothnagel *et al*, 1995).

Recently, Sahn and co-workers (1992) identified a distinct clinical phenotype with epidermolytic and hyperkeratotic features characterized by the development of numerous annular and polycyclic erythematous hyperkeratotic plaques on the trunks and the proximal extremities. These authors pointed out that these peculiar features had not been previously reported in other bullous types of ichthyoses with a histopathology of epidermolytic hyperkeratosis and therefore termed this unique phenotype, annular epidermolytic ichthyosis (AEI). Here we report a second family suffering from the symptoms of AEI and demonstrate the presence of a dinucleotide mutation in keratin 10, which arose *de novo* in the index patient and was later transmitted to an affected daughter.

MATERIALS AND METHODS

Polymerase Chain Reaction (PCR) and DNA Sequencing Genomic DNA was extracted and purified from whole blood as described previously (Rothnagel *et al*, 1992). The 2B region of keratin 10 was amplified from genomic DNA using specific oligonuleotides (5′-AAGTGCTGAAACTGAAATGGTGCC-3′ and 5′-TCTACCCTCTCTCCTCCCTTCCTC-3′) corresponding to bp 4373–4396 and to 4837–4860 of the published

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Abbreviations: AEI, Annular epidermolytic ichthyosis; BCIE, bullous congenital ichthyosiform erythroderma; IBS, ichthyosis bullosa of Siemens.

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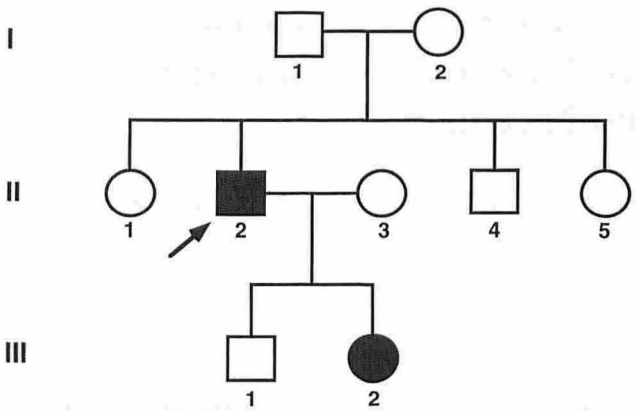

Figure 1. Pedigree of the AEI family. ● and ■ denote affected individuals; ○ and □ denote unaffected individuals; males are represented by *squares* and females by *circles*. The → indicates the proband (individual II.2).

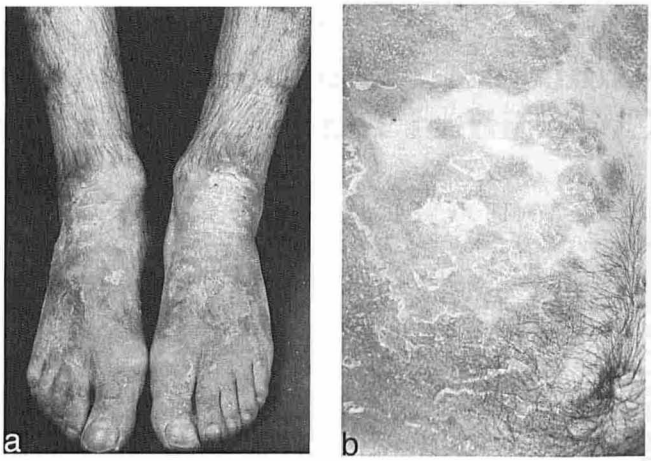

Figure 2. Comparison of the two clinical representations of AEI in the proband. *(a)* Typical appearance of the extremities. Note the keratotic lichenification and superficial erosions over the back of the feet. *(b)* Typical appearance of trunk lesions. Note the annular and polycyclic erythematous plaques with superficial blister formation.

sequence (Rieger and Franke, 1988). The sequencing primer was 5′-ATAAGCGTCACCATACTC-3′, corresponding to bp 4463–4480 of the published sequence (Rieger and Franke, 1988). After PCR amplification, the DNA was captured onto streptavidin-coated magnetic beads via a biotin moiety on one of the oligonucleotides and denatured, and the resultant single-stranded DNA was used as a template in a manual DNA sequencing reaction (Rothnagel *et al*, 1992).

Cloning of PCR Products The amplified DNA containing the 2B region of keratin 10 was purified from a Nu Sieve (FMC Corp., Rockland, ME) gel using the Wizard PCR Prep System (Promega, Madison, WI). The purified PCR product was cloned into a pGEM-T vector (Promega) and subsequently sequenced.

Immunohistochemistry Biopsy specimens were fixed in paraformaldehyde and paraffin embedded. The sections were stained with hematoxylin and eosin for routine histopathology. Deparaffinized sections were predigested with 0.05% trypsin, washed in 1% methanolic hydrogen peroxide, rinsed with phosphate-buffered saline, pre-incubated with 2% bovine serum albumin, and incubated with one of the following primary antibodies: anti-keratin 1 or anti-keratin 10 antibodies (dilution: 1:30, Dianova, Hamburg, Germany), anti-involucrin (dilution: 1:50, Harbor Bio Products, Norwood, MA 02062), anti-filaggrin (dilution: 1:800, Harbor Bio Products), or anti-MIB 1, which recognizes the KI-67 antigen and measures proliferation (dilution: 1:40, Dako, Glostrup, Denmark). The sections were then developed by an indirect immunoperoxidase technique using peroxidase-conjugated goat-anti-mouse or anti-rabbit antibodies (dilution 1:50, Dianova), 0.01% hydrogen peroxide, and 3-amino-9-ethylcarbazole (Sigma, St. Louis, MO). Counterstaining was performed with Harris's hematoxylin (Merck, Darmstadt, Germany).

Electron Microscopy For routine electron microscopy the tissue was fixed in Karnovsky's fixative at 4°C (Karnovsky, 1965), postfixed in 1% osmium tetroxide, and embedded in Epon. Ultrathin sections (60 nm) were cut, mounted on copper grids, stained with uranyl acetate and lead citrate, and subsequently examined in a Philips CM10 electron microscope.

RESULTS

Clinical Features Are Consistent With a Diagnosis of Annular Epidermolytic Ichthyosis The proband is a 33-y-old man, born to unaffected nonconsanguineous parents **(Fig 1)** who consulted us because of an acute aggravation of his skin condition. He had suffered from extensive blistering with the development of erosions since birth. During the first year of life, visible hyperkeratoses developed and he suffered during the following years from bouts of widespread blistering associated with erythema and pruritus until the age of 16 y. Thereafter he was able to control his skin condition with an urea-containing ointment. At the age of 31, new bouts of blistering occurred and he treated himself continually for 2 y with a corticosteroid cream. Physical examination showed hyperkeratotic lichenified plaques with coarse scales forming linear rows located primarily over predilection sites (ankles, dorsum of hands, wrists, and antecubital and popliteal fossae and axillae) **(Fig 2*a*)**. In addition, the trunk and the proximal extremities were covered by numerous migrating, annular, and polycyclic erythematous plaques with scaling borders, and very superficial denudation of the epidermis within these plaques gave rise to superficial erosions **(Fig 2*b*)**. Palms and soles were unaffected. Fungus cultures from the annular plaques were repeatedly negative as was direct staining for hyphal elements.

The patient was treated for the previous use of fluorinated topical ointments with systemic corticosteroids (Prednisolone) in tapering dosages to compensate for steroid withdrawal. He was then started on a treatment regimen of low doses of acitretin, initially with 20 mg daily for several months, then later with 10 mg every other day. In addition, he was treated topically with an ointment containing propylene glycol and 5% urea. Under this regimen, his skin slowly improved and in particular, the annular erythematous lesions completely resolved. During the pedigree work-up, his wife was pregnant with their second child, but they declined to have a prenatal diagnosis. Five months later, their daughter (**Fig 1,** III.2) was born. One day after birth, she developed widespread blistering accompanied by moderate erythema. At the age of 8 wk, the erythema had subsided but a few coin-sized sharply demarcated erosions were still present around the groin and the inner sides of the upper legs. Slight scaling was also noted over the backs of the feet.

Histologic and Ultrastructural Findings Show Typical Features Found in Epidermolytic Hyperkeratosis Biopsies were taken from the trunk of the proband from a site with annular erythematous plaques and from the upper left arm from a region showing marked keratotic lichenification. The biopsy from the annular lesions showed a moderately hyperplastic parakeratotic epidermis with an almost complete absence of keratohyalin granules, broadened rete ridges, and partial thinning of the suprapapillar areas (not shown). The number of mitotic events was increased and some suprabasal mitotic figures were also evident. A few neutrophils were observed within the epidermis including the stratum corneum. A perivascular infiltrate composed of lymphocytes, histiocytes, some neutrophils, and extravasal erythrocytes was present in the upper dermis. Blood vessels were normal. The histology obtained from the keratotic lesion revealed a papillated hyperplastic epidermis with orthohyperkeratosis (not shown). In the non–uniformly expanded granular layer the basophilic keratohyalin granules appeared enlarged and irregularly shaped. The keratinocytes in the mid and upper layers were characterized by ill-defined

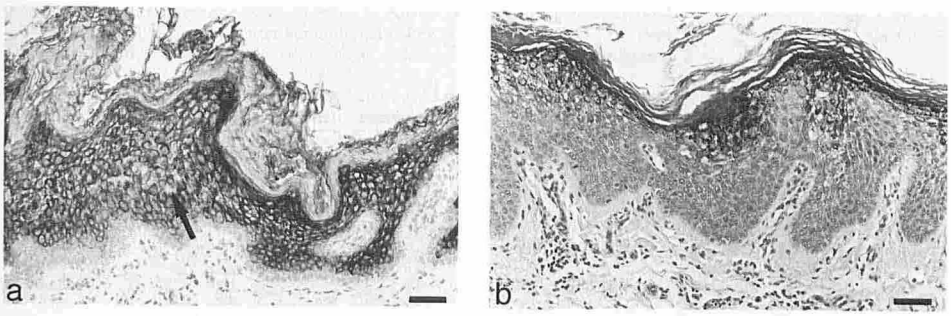

Figure 3. Expression of the differentiation-specific markers in the biopsy from a keratotic lesion. *(a)* Immunohistochemical staining for keratin 10 shows expansion of suprabasal layers. The → indicates an epidermolytic area and the possible site of a future blister. *(b)* Staining for filaggrin expression demonstrating the nonuniform expansion of the granular layer. *Scale bar*, 50 μm.

cell borders and a clear cytoplasm variably containing homogenous eosinophilic bodies and associated with cleft formation. The papillary dermis also contained a discrete perivascular lymphohistiocytic infiltrate.

Immunohistochemistry revealed normal K1 and K10 expression in the suprabasal keratinocytes, but a nonhomogeneous staining pattern was observed in the epidermolytic cells **(Fig 3*a*)**. Involucrin expression was normal (not shown). Staining density of the anti-filaggrin antibody paralleled the expression of keratohyalin granules **(Fig 3*b*)**. Both biopsy specimens showed an increased number of mitotic figures in basal and suprabasal keratinocytes using the MIB 1 antibody (not shown).

Electron microscopy of the keratotic lesion showed abnormal keratin filaments in the suprabasal keratinocytes **(Fig 4)**. Tonofilaments appeared variably aggregated at the cell periphery with large areas of the cytoplasm containing only cell organelles, ribosomes, and some glycogen. Granular layer cells contained enlarged keratohyalin granules embedded in irregularly clumped keratin filaments; their desmosomes were intact and lysis was not apparent.

Sequence Analysis Reveals a Novel Double-Nucleotide Mutation In our initial analysis we sequenced those regions of the differentiation-specific keratins in which disease-associated mutations had previously been documented (Rothnagel, 1996), namely the H1, 1A, and 2B segments of keratin 1 and the 1A and 2B segments of keratin 10. This analysis revealed that two nucleotides were substituted (CG to GA) in codon 388 of keratin 10 in the proband and his affected daughter **(Fig 5*a*)**. Each reaction was repeated and the products sequenced independently to ensure that these sequence changes were not the result of a spurious alteration introduced by the polymerase chain reaction. Notably, unaffected family members did not exhibit these base changes in their DNA and these changes were not observed in the DNA from 50 control unrelated individuals.

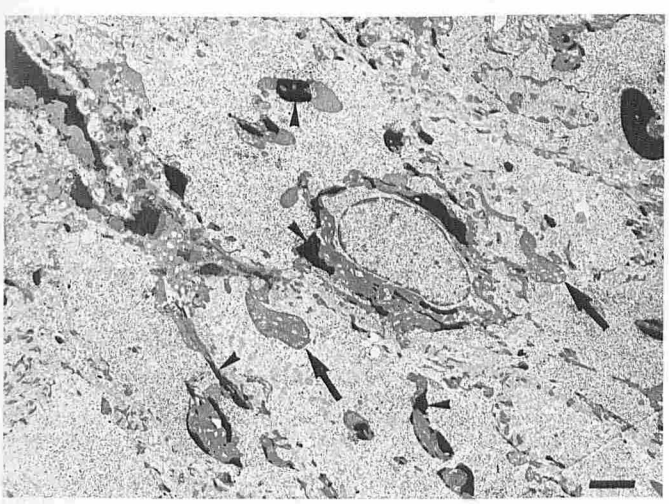

Figure 4. Electron microscopy of lower granular layer cells from the biopsy of the keratotic lesion. Tonofilaments are irregular and highly aggregated (→) with keratohyalin granules (➤) embedded in the keratin filament aggregates. *Scale bar*, 6.25 μm.

The Double-Nucleotide Mutation Is Present in One Allele To ascertain whether these mutations represented a compound heterozygote, both keratin 10 alleles were examined for sequence changes. The region encoding the 2B segment of keratin 10 in the proband was amplified, cloned, and sequenced. This analysis revealed that the double nucleotide mutation (CGA to GAA) occurred in only one allele **(Fig 5*b*)**, resulting in an arginine (R) to glutamate (E) substitution at position 83 (R83E) of the 2B segment. The double-nucleotide mutation deletes a *Taq*I restriction site (TCGA). Therefore, it was possible to confirm loss of the *Taq*I site in one allele by amplification of this region from genomic DNA and digestion with *Taq*I (data not shown).

DISCUSSION

In this study we have identified a novel dinucleotide mutation within the 2B segment of keratin 10 that is associated with a clinical phenotype. The tandem two-nucleotide mutation first arose in the proband and was later transmitted to his daughter. Although we have no functional data proving that this mutation is pathogenic, there is other evidence that strongly suggests that it is. This mutation was only observed in individuals with clinical symptoms and not in unaffected family members. Moreover, the mutation was not observed in any of the 100 normal keratin 10 alleles that were screened as part of this study, and there have been no reports of silent polymorphisms occurring at this site. The affected residue at position 83 of the 2B helical segment is absolutely conserved in all functional type I keratins and is always a basic amino acid in type II keratins and in the other intermediate filament proteins (Conway and Parry, 1988). This conservation of sequence suggests that any change at this position is likely to be deleterious and the substitution of an arginine for a glutamate residue would represent a large alteration in charge at this "e" position of the heptad repeat. Residues in this position are generally thought to form strong ionic interactions with oppositely charged residues on the neighboring molecule in a heterodimer (Conway and Parry, 1988; Steinert *et al*, 1993). Some of these residues, however, may also contribute to higher order interactions between heterodimers. The mutation identified here is postulated to interfere with these interactions, leading to a weakening of the keratin filament network and the subsequent clinical manifestations in these patients.

The tandem double-nucleotide substitution found in this study is the first report of such a mutation in a keratin gene. Most of the disease-associated mutations identified in keratins to date have been single-base substitutions except for three incidences of nucleotide deletions (Rothnagel, 1996). Tandem mutations are rare events, but they have been observed in other genes in both genetic (Winnard *et al*, 1992) and acquired disorders (Blaszyk *et al*, 1994). The genetic events that resulted in the mutation seen in our patients are not known, but it is noteworthy that a CpG dinucleotide is involved inasmuch as mutations at these bases appear to be enhanced due to the spontaneous deamination of 5-methylcytosine in either DNA strand, generating a C to T transition (Cooper and

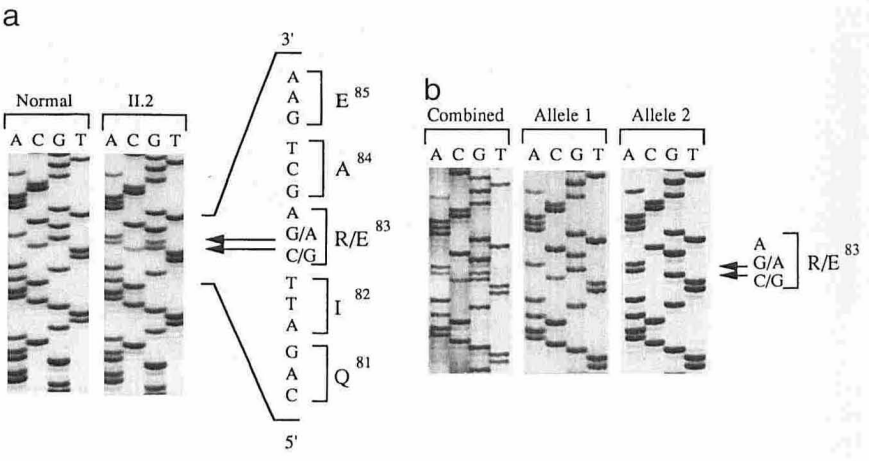

Figure 5. Sequence analysis of the 2B segment of keratin 10 in AEI patients and unaffected family members. *(a)* Direct genome sequencing of the proband (individual II.2 in **Fig 1**) reveals a CGA to GAA double mutation in codon 388. *(b)* Allele-specific sequence analysis of keratin 10 from the proband. Note that both mutations occur in the one allele (Allele 2), resulting in the expression of glutamic acid (E) at position 83 of the 2B segment. Numbering of the amino acid is with respect to the 2B segment of the rod domain.

Youssoufian, 1988). It has also been observed that these sites can exhibit an increase frequency of transversions as well (Bottema *et al*, 1991).

Interestingly, an arginine to cysteine substitution at position 83 of the 2B segment has been observed in keratin 14 in two related patients with epidermolysis bullosa simplex (Chen *et al*, 1995). These patients had a relatively mild form of the disease with symptoms largely confined to their palms and soles. This is consistent with the observation, from both experimental and patient studies, that mutations occurring within the rod domain are generally less deleterious (in terms of phenotypic severity) than those occurring at the ends of the rod domain (Letai *et al*, 1992; Syder *et al*, 1994; Rothnagel, 1996). It is therefore possible that AEI may represent a milder form of BCIE. Of the 20 other mutations identified to date in keratins 1 or 10, only two have occurred within the rod domain but internal to the highly conserved helix-initiating and -terminating motifs (Rothnagel, 1996). One of these, a K100E substitution in 2B of Keratin 10, occurs in patients with very mild symptoms of BCIE (Syder *et al*, 1994), although it is not known whether they had clinical symptoms similar to AEI. The phenotypic severity of the other incident (L103Q in 2B of keratin 10) was not reported (Chipev *et al*, 1994).

From a clinical perspective, AEI represents a unique and recognizable phenotype. The typical histopathology of epidermolytic hyperkeratosis is not present in all affected sites. Thus, our index patient shows psoriasiform histology with an absent granular layer in the biopsy taken from the annular polycyclic lesions (trunk), whereas the typical histopathologic pattern of epidermolytic hyperkeratosis is present in the biopsy taken from a site with maximal scaling (upper left arm). The histologic findings of the annular polycyclic lesions resemble those of the pustular lesions occasionally found in patients with IBS (Steijlen *et al*, 1990). The ultrastructural findings correlate very well with the histopathology and confirm that disease expression in the same patient can be highly variable, ranging from psoriasiform-type lesions to the classic form of epidermolytic hyperkeratosis with the typical perinuclear accumulation of thickened tonofilaments. The clinical heterogeneity displayed by keratin disorders, even between patients with the same underlying mutations, is well documented, but the molecular basis for the variation in phenotypic expression is not well understood (DiGiovanna and Bale, 1994). It has been suggested that this heterogeneity may be attributable to the idiosyncratic expression of mutant proteins in certain individuals or to the cellular and/or tissue response to abnormal keratin filaments or to epigenetic causes such life style and environment (Rothnagel *et al*, 1994; Rothnagel, 1996).

Until this study, the question of whether AEI is a distinct genodermatosis or a phenotypic variant of one of the bullous ichthyosiform erythrodermas remained unresolved. Our molecular data provide evidence that AEI is a clinical variant of BCIE. It should be noted however, that AEI runs a much milder course than is usually found in patients with BCIE. Nevertheless, because we identified keratin 10 mutations in these patients, AEI should not be considered a distinct disorder but rather a variant of BCIE.

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