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C Baudoin, … , G Meneguzzi, J P Ortonne

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Research Article

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Herlitz Junctional Epidermolysis Bullosa Keratinocytes Display Heterogeneous Defects of Nicein/Kalinin Gene Expression

Christian Baudoin,* Corinne Miquel,* Claudine Blanchet-Bardon,‡ Claudio Gambini,§ Guerrino Meneguzzi,* and Jean-Paul Ortonne*

**INSERM U385, UFR de Médecine, 06107 Nice; ‡Clinique des Maladies Cutanées et Unité de Recherche, Diagnostic Anténatal en Dermatologie, Hôpital Saint-Louis, 75 Paris, France; and §Servizio d'Istologia, Ospedali Galliera, 16128 Genova, Italy*

Abstract

Previous studies have correlated the Herlitz junctional epidermolysis bullosa (H-JEB) to an altered expression of the basement membrane component nicein/kalinin. This heterotrimeric glycoprotein appears to be present in H-JEB tissues in an abnormal form, because a number of antibodies specific to the protein either do not react with or weakly stain the epidermal basement membranes of most of the patients. With cDNA probes encoding each subunit of nicein and polyclonal antibodies raised against bacterial fusion polypeptides corresponding to the individual chains of the protein, we have molecularly analyzed the expression of nicein in H-JEB tissues and cultured keratinocytes. By immunohistochemistry, Northern blot, and protein analysis, we show a defective synthesis of one of the nicein subunits in six cases of H-JEB from five different consanguineous families. In two patients, the disease correlates with an impaired synthesis of the nicein B2 (nic B2) chain, in three others with that of the B1 (nic B1) chain, and in a sixth patient with that of the heavy A (nic A) chain. In this report, we thus demonstrate that H-JEB is a genetically heterogeneous disease and we provide strong evidence that the genes of nicein are the candidates for this genodermatosis. (*J. Clin. Invest.* 93:862–869.) Key words: nicein/kalinin • basement membrane • junctional epidermolysis bullosa • dermal-epidermal junction • hemidesmosomes

Introduction

Hereditary epidermolysis bullosa (EB)[1] is a group of blistering skin diseases characterized by tissue separation within the dermal-epidermal basement membrane zone (1, 2). In the simplex forms of EB, intraepidermal tissue separation occurs at the level of basal keratinocytes (1). The different clinical manifestations of EB simplex have been recently attributed to mutations on the genes encoding keratins 5 and 14, which are expressed by basal keratinocytes (3–5). In the dystrophic scarring forms of EB, the cleavage occurs within the papillary dermis, below the basal lamina at the level of the anchoring fibrils (1). Several lines of evidence identified type VII collagen, the main component of anchoring fibrils, as the candidate gene for dystrophic EB (6, 7). Subsequent genetic analysis of affected kindreds and the identification of point mutations in the collagen VII DNA sequences strongly support the involvement of the gene in the pathology (8–10). The junctional forms of EB (JEB) result from tissue separation within the lamina lucida of the dermal-epidermal basement membrane. Several varieties of JEB have been described (1). The generalized form, or the Herlitz's variant (H-JEB), presents the most severe clinical manifestations with widespread blistering of skin and mucous membranes. In most cases, H-JEB is fatal early in life. The non-Herlitz variants are characterized by continuous blistering from birth or early infancy that does not interfere with normal development and life span. All forms of JEB are autosomal recessive.

Ultrastructural studies of JEB skin have demonstrated abnormalities in hemidesmosome (HD) shape and number (11). HD are cellular structures that appear as electron microscope thickenings making connection with intermediate filaments (12, 13). They are located at the interface of basal epithelial cells with the underlying dermis, and are presumed to function as part of an epithelial cell–substratum attachment device (14). Considerable progress has recently been made in unraveling the molecular composition of HD (12). Domains of the bullous pemphigoid antigens 1 (BPA G1) and 2 (BPA G2) (15, 16) and those of a 200-kD polypeptide (17) reside in the hemidesmosomal plaque to which keratin bundles attach. An additional high molecular mass component (HD1) is located on the cytoplasmic side of HD (18). Integrin $\alpha 6\beta 4$ is also found in the HD (19–22), and the cytoplasmic domains of the protein may play a role in the junctional connections of hemidesmosomal structures to the cytoskeleton (12).

The bridge between the HD and the lamina densa region of the basement membrane is provided by the anchoring filaments (12). Noncollagenous proteins immunolocalizing with these structures have been identified. They comprise nicein/BM-600 (23, 24) and kalinin (25), which have been demonstrated to be the same protein (26). Nicein/kalinin is a heterotrimeric protein containing three nonidentical subunits, covalently linked by disulfide bonds (24, 25). The molecular masses of the three chains as reported by two different groups are, respectively, 100/105 kD for the lightest subunit (nic B2), 125/140 kD for the second chain (nic B1), and 150/165 kD for the heaviest subunit (nic A). It has been documented that maturation of nicein/kalinin occurs extracellularly in two steps from a high molecular mass precursor (27). cDNA encoding for the three subunits were recently isolated, and their

Address correspondence to Dr. Jean-Paul Ortonne, INSERM U385, UFR de Medecine, Avenue de Vallombrose, 06107 Nice Cedex 02, France.

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1. *Abbreviations used in this paper:* EB, epidermolysis bullosa; H-JEB, Herlitz junctional epidermolysis bullosa; HD, hemidesmosome; nic A, nicein A chain; nic B1, nicein B1 chain; nic B2, nicein B2 chain; pAb, polyclonal antibody.

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sequences revealed that nicein is a laminin-like protein (28–30). nic B2 is homologous to the recently reported laminin-like chain B2t (31). Laminin and nicein both display a cross-shaped conformation, nicein presenting the short arms of the cross reduced in size (27). The novel laminin variant, K-laminin containing 220-kD B1 and 210-kD B2 laminin chains, as well as a 190-kD novel chain, is also associated with these hemidesmosomal structures (32). Less well-characterized anchoring filament components are the protein epiligrin and the 19-DEJ-1 (33, 34). Epiligrin antibodies precipitate peptides with electrophoretic migrations similar to those of both nicein/kalinin and K-laminin (35). The molecular mass of the 19-DEJ-1 antigen is unknown (34).

No large scale abnormalities in the BPA G1 gene were found by Southern analysis of H-JEB genomic DNA (36). Immunohistological studies have demonstrated that $\alpha 6\beta 4$ (37) is normally expressed by H-JEB basal keratinocytes. $\alpha 6\beta 4$ nevertheless appears more defocalized at central basal areas of the cells (38). On the contrary, the expression of nicein/kalinin is dramatically altered (39–42). Antibodies directed against nicein do not react with the epidermal basement membranes of most of H-JEB patients and abnormal staining can also be observed in the non-H-JEB patients (40, 41). Indirect immunofluorescence studies of H-JEB skin using polyclonal antibodies (pAbs) to the individual purified subunits of nicein suggest that the protein is present but structurally altered (43). However, because of the cross-reactivity between pAbs directed against the nic B2 and nic A subunits, the cause underlying the undetectability of nicein in H-JEB skin was not determined.

The availability of cDNAs encoding for the three nicein subunits allowed the production of pAbs directed against fusion proteins corresponding to the nic B2 and nic A chains of human nicein. These antibodies and the corresponding nicein cDNA probes have been used to follow the synthesis of the different chains of nicein in skin and cultured keratinocytes of H-JEB patients.

Methods

Tissues. Skin biopsies were from six H-JEB patients from five different families: two from 20–21-wk-old fetuses (Gf, F) and four from newborns (L, Gb, B, S). In all cases, H-JEB was diagnosed according to the revised criteria for subtyping EB (1). The level of cleavage in all lesional skin samples was confirmed according to immunofluorescence mapping of VLA-6, laminin, and type IV collagen. Transmission electron microscopy showed a dermo-epidermal split in the lamina lucida. All samples presented abnormal HD. The skin biopsies did not react with GB3 or 19-DEJ-1 mAbs (44). Controls were normal fetal skins from 18–24-wk-old fetuses and from adult healthy volunteers.

Antibodies. For the characterization of basement membrane components, the following mAbs or pAbs were used: mAb GB3 (hybridoma culture medium; working dilution, 1:2) (45) rabbit pAb anti-human collagen IV (working dilution, 1:2; Institut Pasteur, Paris, France), rabbit pAb anti-human collagen VII (working dilution, 1:10), goat pAb anti-mouse laminin (working dilution, 1:50; Sigma Chemical Co., St. Louis, MO) mAb anti-EBA antigen (working dilution, 1:50), mAb LH 7:2 (working dilution, 1:10), mAb 19-DEJ 1 (working dilution, 1:50), mAb GB36 (working dilution, 1:2). Three sera from patients with herpes gestationis (working dilution, 1:2–1:5) and five from patients with bullous pemphigoid (working dilution, 1:50–1:100) were also used.

The antinicein pAbs SE 144 and SE 85 were raised against fusion proteins derived from cDNAs encoding the central domain of the nic B2 subunit and the COOH-terminal domain of the nic A subunit, respectively (27, 30). The pAb α125 specifically recognizes the nic B1 subunit of nicein (43). The mAb K 140 specific to the nic B1 subunit of nicein/kalinin (31) and the mAb BM 165 specific to the nic A chain of nicein/kalinin, its 200-kD precursor (25), and to the 190-kD subunit of K-laminin have also been used (31). Nonimmune sera (normal human sera and preimmunized rabbit sera or culture medium of P3-NS1-Ag4-1 (NS 1 cell line) were used as control.

Cell culture. Split thickness epidermis was separated from dermis by dispase treatment at 37°C. Keratinocytes were dissociated in trypsin 0.25% at 37°C, and plated on a feeder of irradiated 3T3 cells (ICN, Costa Mesa, CA) (46). Keratinocytes were grown in a 1:1 mixture of DMEM and Ham's F12 (GIBCO BRL, Eragny, France) containing 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine, 10 μg/ml of penicillin and streptomycin, 10 ng/ml EGF, 100 pM choleratoxin, 400 ng/ml hydrocortisone, 5 μg/ml transferrin, 180 μM adenine, and 20 pM T3 (47). H-JEB keratinocytes were expanded after gentle dissociation in 0.05% trypsin, 0.02% EDTA.

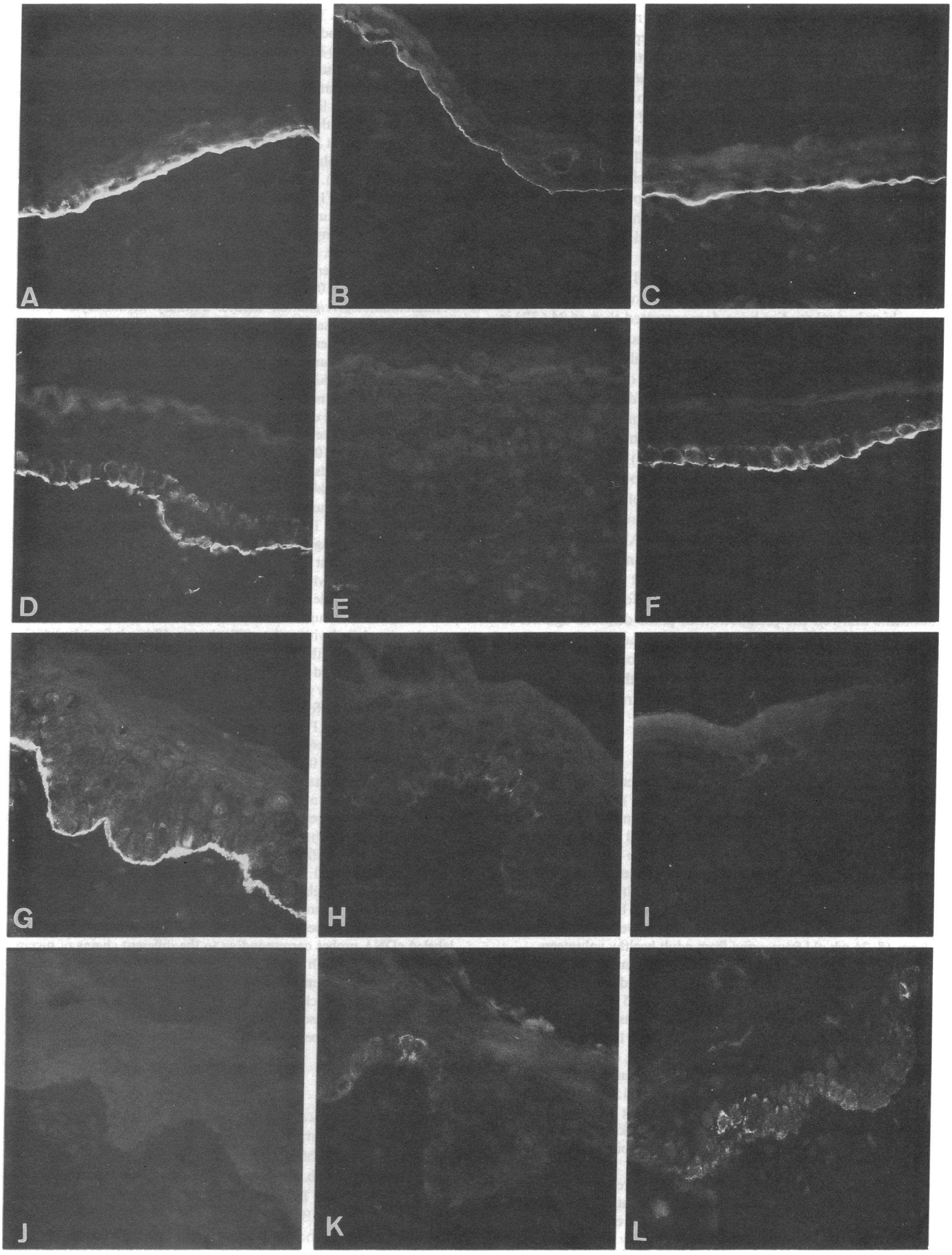
Radioimmunoprecipitation assays. Primary keratinocytes (0.6×10^6 cell/well) were seeded in 9-cm^2-well plates and allowed to attach for 12 h. Cells were then labeled for 24 h in 1.5 ml of methionine- and cysteine-deficient culture medium (GIBCO BRL) supplemented with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine (Amersham International, Amersham, England). Culture medium was centrifuged at 12,000 rpm to remove cells and debris, and TCA-precipitable radioactivity was counted. Preclearing was performed by adding aliquots of each sample ($\sim 5 \times 10^6$ TCA-precipitable cpm) to a pellet of protein A–Sepharose 4B beads (Pharmacia SA, Uppsala, Sweden) precomplexed with either rabbit anti-mouse IgG (Dakopatt, Copenhagen, Denmark) or preimmune rabbit serum, respectively, for further use with mAbs and pAbs. The reaction mixtures were incubated for 1 h at 4°C under rotatory agitation. Precleared aliquots were then centrifuged, and supernatant incubated with mAb K 140 (nic B1) at a final dilution of 1:100 or with SE 144 (nic B2) at a final dilution of 1:40 overnight at 4°C under rotatory agitation. For control conditions a preimmune rabbit serum was used at a final dilution of 1:40. Samples were then incubated with pellets of Sepharose 4B beads for 1 h at 4°C. After centrifugation the supernatant was removed and the pellet washed once with cold Tris-buffered saline, 0.1% NP-40, briefly vortexed, then extensively washed four times with radioimmunoprecipitation buffer (RIPA buffer: 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 250 μM PMSF, 1 mM *n*-ethylmaleimide, 2 mM L-methionine, 2 mM L-cysteine, 0.3% NP-40, 0.05% Triton X-100, 0.3% sodium deoxycholate, 0.1% BSA) containing 0.1% SDS (31), and then with RIPA buffer containing 0.5 M NaCl, and rinsed once with TBS. Immunoprecipitated materials were then analyzed on a 6% SDS-PAGE gel electrophoresis (48).

Northern blot analysis. Total RNA was prepared from H-JEB and normal cultured keratinocytes according to the method of Schomczyski and Sacchi (49). RNA was subjected to electrophoresis on 1.2% denaturing agarose gels containing 1.2 M formaldehyde and transferred onto nylon membrane (Hybond N; Amersham International). Membranes were subsequently hybridized at high stringency with ^{32}P-labeled cDNA probes corresponding to the different chains of nicein and then exposed 16 h on Hyperfilm MP (Amersham International) with intensifying screens.

The radiolabeled cDNA probes PCR 1.3 (27), KAL-5.5C (28), and NA1 (29) were used to detect the mRNA for the nic B2, nic B1, and nic A chains, respectively.

Results

Immunohistochemical detection of nicein chains in normal and H-JEB tissues. Immunofluorescence experiments were performed on samples of human epidermis using pAbs specifically directed against each single chain of nicein (Fig. 1 and Table I). Presence of the nic B2 subunit was determined with pAb SE 144, that of the nic B1 subunit with pAb Pα 125 and/or mAb K-140, and that of the nic A subunit with pAb SE 85. In normal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skin, all the antibodies labeled the epidermal basement membrane (Fig. 1, *A–C*). In most basal keratinocytes, pAb Pα 125 (nic B1) (Fig. 1 *B*) and pAb SE 85 (nic A) (Fig. 1 *A*) gave a cytoplasmic staining, whereas pAb SE 144 (nic B2) reacted more faintly with the cytoplasm of only some of the basal cells (Fig. 1 *C*). Results obtained with patients Gb and Gf were consistent with observations made on patient F. Patients A and L had similar patterns of reactivity to the antinicein antibodies. Patient S presented a distinct immunological reactivity. Representative results are shown in Fig. 1, *D–I*.

In patients Gb, Gf, and F, the pAb Pα 125 (nic B1) failed to react with both the dermal-epidermal junction and the cytoplasm of epidermal keratinocytes, while staining with the pAbs SE 144 (nic B2) and SE 85 (nic A) was indistinguishable from that of normal controls, with the exception of the cytoplasmic staining, which was markedly stronger (Fig. 1, *D* and *F*). This result suggests an impairment of the synthesis and/or the secretion of the nic B1 chain, since the other chains of nicein are apparently normally expressed. Tissue from patients A and L exposed to pAb SE 144 (nic B2) showed no immunoreactivity (Fig. 1 *J*). pAb Pα 125 (nic B1) also did not label the epidermal basement membrane of these patients, but presence of the nic B1 subunit was detected in isolated basal keratinocytes in patient A (Fig. 1 *H*). In both patients, the expression of the heavy chain (nic A) of nicein was comparable to that of healthy controls (Fig. 1 *G*). Clearly, in these two cases only the expression of the nic B2 chain was completely absent. Both the pAbs SE 85 (nic A) (Fig. 1 *J*) and Pα150 (nic A) (data not shown) did not reveal any specific staining; a cytoplasmic reactivity was obtained with pAb Pα 125 (nic B1) (Fig. 1 *K*) and pAb SE 144 (nic B2) (Fig. 1 *L*), which indicates a low rate of synthesis of the light chains of nicein. Thus, in this case, only the expression of the nic A chain is undetectable. All other antibodies tested (see Methods) stained the epidermal basement membrane normally (data not shown).

Expression of nicein mRNA in H-JEB keratinocytes. To check whether the altered expression of nicein subunits detected by immunofluorescence reflected possible structural alterations of nicein chains or absence of nicein chains, the expression of nicein genes was examined by Northern blot analysis of mRNA purified from primary cultures of keratinocytes obtained from the six H-JEB patients. Total mRNA was fractionated by agarose gel electrophoresis, blotted onto nitrocellulose, and subsequently hybridized to the nick-translated nicein cDNA probes.

Consistent with the immunofluorescence observations in patients Gb and F, the probe specific for nic B1 chain failed to detect the corresponding mRNA, whereas the nic B2 chain and nic A chain mRNA were normally expressed (Fig. 2). However, the nic B1 chain mRNA could be detected as a very faint band of 4 kb in cultured keratinocytes from an affected fetus (Gf) of the G family (Fig. 2 *B*). This suggests that its lack of detection in the other patient of the G family (Gb) was due to a strong decrease in transcription or mRNA stability rather than to a total absence. These results not only confirmed the immunofluorescence observations, indicating a possible altered expression of the nic B1 chain, but they further demonstrated an impairment of transcription or/and an instability of the mRNA.

In the case of patient A and consistent with immunofluorescence data, the messengers corresponding to the chains nic B1 and nic A were normally expressed as strong bands of 4 and 6 kb, respectively, while the nic B2 mRNA were undetectable (Fig. 2 *C*). In patient L, where antibodies to the nic B2 and nic B1 chains gave no immunoreactivity, only the nic B2 chain mRNA was not detected. Thus, Northern blot analysis and morphologic observations both demonstrate that the synthesis of the nic B2 chain is specifically impaired in these two cases.

In patient S, the mRNA corresponding to all the three chains of nicein were present, but the mRNA for the nic A chain was greatly reduced (Fig. 2 *A*), whereas the expression of the mRNA for the other two chains appeared normally expressed. No appreciable changes in the size of the different mRNA were seen.

Synthesis of nicein chains. In normal human keratinocytes (Fig. 3, lanes *2* and *3*), mAb K 140 (nic B1) and pAb SE 144

Table I. Immunofluorescence Data

	Nicein chain					
	nic A		nic B1		nic B2	
Patients	EC	IC	EC	IC	EC	IC
Gf and Gb	++	++	−	−	++	++
F	++	++	−	−	++	++
A	++	+	−	+*	−	−
L	++	+*	−	−	−	−
S	−	−	−	+*	−	++
Controls	++	+	++	+*	++	+*

Scored immunofluorescence with pAbs SE 85 (nic A), Pα 125 (nic B1), and SE 144 (nic B2) in H-JEB skin and in normal controls. Absence of fluorescence (−), detectable levels of staining (+), and strong fluorescence (++) are indicated. EC, extracellular staining (labeling of the dermo-epidermal junction). IC, cytoplasmic staining of epidermal basal keratinocytes. * Staining of scattered epidermal keratinocytes.

Figure 1. Immunofluorescence analysis with the polyclonal SE 85 (anti-nic A), SE 144 (anti-nic B2), and Pα 125 (anti-nic B1) of healthy (*A–C*) and H-JEB fetal skin (*D–L*). (*A–C*) Control fetal skin. All the antibodies label the epidermal basement membrane. pAb SE 85 (nic A) also stains basal cells (*A*). pAbs Pα 125 (nic B1) (*B*) and SE 144 (nic B2) (*C*) give a faint cytoplasmic staining restricted to isolated keratinocytes. (*D–F*) Patient Gf. pAb SE 85 (nic A) gives the typical linear pattern of fluorescence at the dermal epidermal junction; a strong cytoplasmic staining of basal keratinocytes is also observed (*D*). pAb Pα 125 (nic B1) does not label the epidermal basement membrane (*E*). With pAb SE 144 (nic B2), the staining of the dermal epidermal junction is comparable to that obtained in controls although with a stronger cytoplasmic reaction with basal keratinocytes. (*G–I*) Patient A. pAb SE 85 (nic A) reacts as in control skin (*G*). With Pα 125 (nic B1), the epidermal basement membrane is not reactive, but scattered basal keratinocytes show a cytoplasmic staining (*H*). No reactivity is detected with pAb SE 144 (nic B2) (*I*). (*J–L*) Patient S. None of the three pAbs, SE 85 (*J*), Pα 125 (nic B1) (*K*), and SE 144 (nic B2) (*L*), stain the dermal-epidermal junction. pAbs Pα 125 (nic B1) (*K*) and pAb SE 144 (nic B2) (*L*) decorate the cytoplasm of isolated basal keratinocytes. With pAb SE 144 (nic B2), this staining is stronger than in control skin.

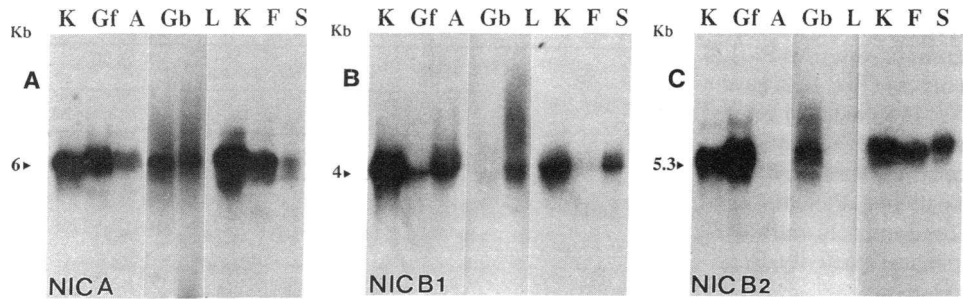

Figure 2. Northern blot analysis of human normal (*K*) and H-JEB keratinocytes from six patients. 10 μg of total RNA purified from primary cultures of keratinocytes was subjected to electrophoresis on agarose gels, blotted to a nitrocellulose sheet, and successively probed with ^{32}P-labeled cDNAs for the nicein chains nic A (*A*), nic B1 (*B*), and nic B2 (*C*), respectively. (*A*) In patient S the mRNA for the nic A chain gives a signal reduced in intensity. (*B*) In patients Gb and Gf, the mRNA specific for nic B1 is undetectable or its amount strongly reduced. (*C*) In patients A and L the mRNA for the nic B2 chain is not detected.

(nic B2) immunoprecipitate the mature form of nicein present in conditioned culture media, as documented by the detection of the three subunits of the protein at the molecular masses of 105, 140, and 160 kD, respectively, which approximate those previously reported for the mature forms of nicein and kalinin. A faint band at 150 kD was also present, consistent with unprocessed B2 chain previously reported (27). An additional band at 250 kD was visible in all the samples including controls. The bands corresponding to the nicein subunits were not observed when radioimmunoprecipitation was performed with the control preimmune serum (Fig. 3, lane *1*), while the band at 250 kD was clearly detectable in control sample from H-JEB keratinocytes from all the five patients studied (patients L and Gf; Fig. 3, lanes *4* and *7;* not shown for the three other patients) and, faintly, in normal keratinocytes. It was thus concluded that the 250-kD band is nonspecific and presumably be due to fibronectin.

In patient L, mAb K 140 immunoprecipitated four polypeptides of 200, 185, 160, and 138 kD. Consistent with the results of Northern blot analysis, the 105-kD nic B2 chain was not detectable. In parallel radioimmunoprecipitation assays performed with pAb SE 144, which recognizes the nic B2 subunit, no specific band was immunoprecipitated (Fig. 3, lane *6*). A similar immunoprecipitation pattern was observed in experiments with cultured keratinocytes from the patient A (data not shown). These data definitely confirm that in these patients synthesis of nic B2 chain is impaired.

In patient Gf, the mAb K 140 specific to the nic B1 subunit, whose mRNAs are undetectable in keratinocytes from this individual, failed to immunoprecipitate any specific bands (Fig. 3, lane *8*). Conversely, with pAb SE 144 (nic B2), at least nine bands were observed: major bands of 230, 200, 160, 155, 105, 85, and 80 kD and two faint bands at 175 and 125 kD (Fig. 3). Only the band corresponding to the nic B1 chain at 140 kD was not detectable. A similar pattern was observed with patient F (data not shown). The involvement of the Nic B1 was thus clearly demonstrated.

In patient S, where no immunoreactivity was observed at the dermal-epidermal junction, whatever the antibody used, while pAb SE 144 (nic B2) and Pα 125 (nic B1) detected a cytoplasmic staining in basal keratinocytes (Fig. 1, *J–L*), both pAb 144 (nic B2) and mAb 140 (nic B1) immunoprecipitated the three nicein chains. As in the other H-JEB patients, the bands corresponding to the nicein chains were reduced in intensity, compared with the counterparts from healthy donors. In all cases bands similar to those found in the culture medium were seen in the cell extract from the H-JEB keratinocytes (not shown).

Discussion

We previously reported that most H-JEB patients present a widespread defect of nicein. This was demonstrated by the lack of reactivity of H-JEB skin and epithelia to the mAb GB3 (41). Since nicein could not be immunoprecipitated from cultured H-JEB keratinocytes with these antibodies, it was suggested that the abnormal expression of the protein could result from intrinsic defects of H-JEB epithelial cells (41). The molecular

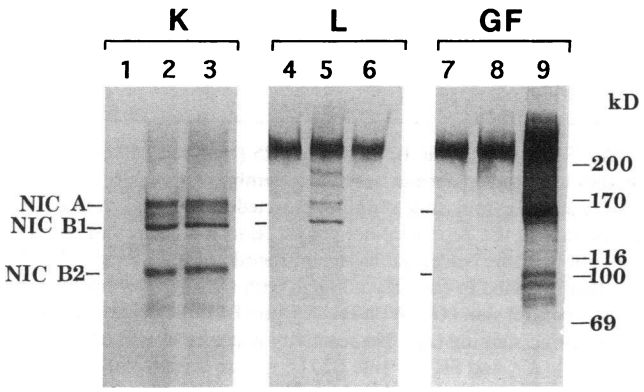

Figure 3. Immunoprecipitation of radiolabeled nicein from culture medium conditioned by human normal (*K*) and H-JEB keratinocytes. Cultures of normal foreskin (lanes *1–3*) and H-JEB keratinocytes from patients L (lanes *4–6*) and Gf (lanes *7–9*) were labeled for 24 h with [^{35}S]methionine and [^{35}S]cysteine. Medium was immunoprecipitated with mAb K 140 (*NIC B1*) and (lanes *2, 5,* and *8*), pAb SE 144 (*NIC B2*) (lanes *3, 6,* and *9*), or with preimmune rabbit serum (lanes *1, 4,* and *7*). Immunoprecipitated proteins were submitted to SDS-PAGE. In controls, bands corresponding to the chains of nicein are clearly detected (lanes *2* and *3*). In patient L, reaction with mAb K 140 does not precipitate the 105-kD subunit (*NIC B2*) of nicein (lane *5*), and no immunoprecipitation band is detected with pAb SE 144, which is specific to the nic B2 chain (lane *6*). In patient Gf, no band corresponding to the nic B1 chain is immunoprecipitated by the pAb SE 144 (lane *9*). No nicein chain is recognized by the anti-nic B1 mAb K 140. The molecular masses of the migration markers are indicated.

Table II. Summary of Immunofluorescence Data, mRNA, and In Vitro Nicein Chains Synthesis

	nic A			nic B1			nic B2		
	Protein		mRNA	Protein		mRNA	Protein		mRNA
Patients	IF	RIP	NB	IF	RIP	NB	IF	RIP	NB
Gf	EC.IC	+	+	−	−	+	EC.IC	+	+
Gb	EC.IC	ND	+	−	ND	−	EC.IC	ND	+
F	EC.IC	+	+	−	−	−	EC.IC	+	+
A	EC.IC	+	+	IC	+	+	−	−	−
L	EC.IC	+	+	−	+	+	−	−	−
S	-	+	+	IC	+	+	IC	+	+

IF, immunofluorescence (−, absent staining; EC, extracellular staining; IC, intracellular staining). RIP, radioimmunoprecipitation (+, detected; −, undetectable). NB, Northern blot (+, detected; −, undetectable). ND, not done.

basis of such an abnormality remained unresolved. Using cDNA probes encoding each subunit of nicein and polyclonal antibodies raised against bacterial fusion proteins corresponding to each individual chain of nicein, we have further analyzed six cases of H-JEB from five different families.

Information obtained from immunofluorescence studies of involved skin, radioimmunoprecipitation of incubation medium, and extracts of cultured H-JEB keratinocytes, and Northern blot analysis, consistently demonstrate that in three patients the disorder correlates with an impaired synthesis of the nic B1 chain, in two others with that of the nic B2, and in a sixth patient with that of the nic A (Table II). It can thus be concluded that in H-JEB the impaired expression of a mature nicein is consequent to a defective synthesis of one of its subunits and that the dermatosis is genetically heterogeneous. This finding is not unexpected considering the fact that nicein comprises three different subunits synthesized by distinct genes.

In the case of patients where the absence of one of the light subunits of nicein was documented by immunofluorescence and Northern blot analysis, immunoprecipitation of keratinocyte culture medium always confirmed the absence of synthesis of the polypeptide, while secreted forms of proteins with electrophoretic migration corresponding to the other two subunits were always identified. Other aberrant nicein proteins were also specifically recognized by the antinicein antibodies, but the corresponding bands presented abnormal electrophoretic migration patterns, different from those expected for nicein subunits. In one case (patient S), the pathology was associated with a lack of immunoreactivity to the anti-nic A antibody. This correlated with a decrease in the steady state level of the corresponding mRNA. Immunoprecipitation of culture medium conditioned by the keratinocytes of this patient with antibodies to the light chains of nicein detected large amounts of the three chains of nicein. It is thus clear that all the three chains are produced and secreted as a complex in this patient. Because the apparent molecular mass of each subunit is consistent with what is expected, conditions may exist in vivo, but not in culture, that account for instability or degradation of the nic A chain leading to undetectability in the skin.

Our results show no appreciable changes in the size of nicein mRNAs in any of the H-JEB families. In patients affected in the expression of one of the light chains of nicein, the amount of the corresponding messenger was dramatically low. Whether the abnormal concentration of such mRNAs is due to a decrease in the synthesis or to a higher decay rate has not been determined. Among the reasons that could lead to such a situation, one should consider the presence of mutations in the promoter regions of the affected gene, a decreased stability of the transcript due to mutations affecting splicing sites, nonsense mutations occurring before the penultimate exon (50), or mutations/alterations of regulatory genes (51).

Nicein is a laminin-like molecule. It has been demonstrated from in vitro analysis of purified fragments that laminin assembly involves the formation of a stable coiled–coil B1-B2 intermediate to which the A chain subsequently interacts to form a more stable triple coiled–coil molecule (52). It has not yet been determined if the assembly of nicein and laminin are analogous processes, but the finding that a complex formed by a heavy chain and a light chain of nicein are immunoprecipitated from the culture medium of H-JEB keratinocytes suggests that, contrary to the model proposed for laminin, in the case of nicein formation of a B1-B2-like dimer, it is not a prerequisite for extracellular secretion. However, from immunoprecipitation data, the presence of a truncated third chain of nicein in H-JEB cells cannot be formally excluded, but, considering the results of the Northern blot analysis, this possibility seems unlikely. On the other hand, a release of a dimer in the culture medium consequent to cell lysis cannot be ruled out.

The nic A chain seems to interact indifferently with either nic B1 or nic B2, because in patients L and Gf this chain appears assembled either with the nic B1 chain or the nic B2 chain, respectively. A complex missing a light chain seems, nevertheless, unstable. In fact, the amount of immunoreactive protein detected at the dermal-epidermal junction of biopsies from patients A, L, Gf, and F is exceedingly low compared with normal controls. However, the amount of nicein chains immunoprecipitated from keratinocyte medium conditioned by keratinocytes of these patients was almost comparable to that of normal keratinocytes. Moreover, a degradation of the heterodimers is suggested by the immunoprecipitation of peptides with electrophoretic migrations different from those found in medium conditioned by normal cells. For instance, the cells from patients Gf and L synthesize two polypeptides with an apparent molecular mass of 175/180 and 200 kD, which are sizes comparable to those of the precursor of the A chain of nicein (31). The relative abundance of these two proteins could be explained by an impairment of the maturation process and/or an inefficient excretion, either as monomers or as dimeric complexes involving the another chain of nicein. This would also explain the presence of the intracellular fluorescence found in H-JEB keratinocytes using antibodies to nic B1 and nic A.

These studies clearly demonstrate the involvement of nicein in the pathogenesis of H-JEB and support the idea that genes for nicein are the candidate for this disorder. However, involvement of other molecules in the etiology of the disease cannot be excluded, since abnormal expression of 19 DEJ-1 antigen has also been observed in patients affected by H-JEB and non-H-JEB subtypes. Further evidence favoring the involvement of nicein in this disorder will be provided by the linkage analysis in the kindreds of the patients studied in this work.

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