Acta Derm Venereol 2014; 94: 307–311

CLINICAL REPORT

T-lymphocytes are Directly Involved in the Clinical Expression of Migratory Circinate Erythema in Epidermolysis Bullosa Simplex Patients

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Epidermolysis bullosa simplex with migratory circinate erythema (EBS-MCE) is a rare EBS subtype characterised by migratory blistering lesions that resolve with brownish pigmentation. It is caused by a recurrent readthrough mutation, c.1649delG, in the tail of keratin 5. Here, we report a child with EBS-MCE and investigated the immunologic mechanisms underlying the migratory lesions in this patient. A skin biopsy from the patient from an active border of an erythematous lesion was used for the immunohistochemical characterisation of the inflammatory infiltrate and for TUNEL assay to detect apoptotic cells. We found abundant $CD4^+$ and $CD8^+$ T lymphocytes infiltrating the papillary dermis and lining the dermal–epidermal junction. A number of these cells expressed the activation marker CD69. $CD83^+$ dendritic cells were present both in the epidermis and papillary dermis. Finally, TUNEL staining showed apoptosis of basal and suprabasal keratinocytes. These findings suggest a critical role of the cellular immunity in determining the EBS-MCE phenotype. ***Key words: genodermatosis; intermediate filaments; mottled pigmentation; nonhelical tail domain.***

Accepted May 21, 2013; Epub ahead of print Oct 8, 2013

Acta Derm Venereol 2014; 94: 307–311.

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Epidermolysis bullosa simplex (EBS) is a group of inherited skin diseases characterised by intraepidermal blistering. According to the level of blister formation, EBS is distinguished in the basal and suprabasal type. The latter refers to less common phenotypes associated with split in the suprabasal epidermal layers (1). Basal EBS (hereafter defined as EBS) is the classical type, which results from cytolysis of basal keratinocytes and cell fragility throughout the basal layer upon mechanical trauma. EBS is mainly caused by autosomal dominant mutations in the genes *KRT5* and *KRT14* encoding keratins 5 (K5) and K14 (1, 2). Heterodimers of K5 and K14 form the keratin intermediate filaments (KIF) of the basal keratinocyte cytoskeleton. In EBS, skin fragility is thought to result from a defective assembly, growth and impaired stability of KIF (3, 4). Mutations, usually missense substitutions, cluster in distinct domains of keratins and the severity of clinical manifestations seems to correlate with the localisation of these mutations (5–7). In the most severe form, the Dowling-Meara subtype (EBS-DM; MIM 131760), mutations are almost all located in the highly conserved ends of the alpha-helical rod domain of K5 and K14, whereas in the mildest variants, the 'localised EBS' (previously Weber-Cockayne; MIM 131800) and the 'EBS, other generalised' (previously Köebner; MIM 131900), mutations affect less conserved sequences of the alpha-helical rod domain, such as the linker regions. Unusual EBS variants have also been recognised that are caused by recurrent mutations outside the rod domain, in the head and tail of K5 (1, 2). These mutations are believed to impair keratin filament elongation resulting in skin blistering and defective melanin granule aggregation. Mutation p.P25L in the K5 head domain is the most frequent substitution identified in the EBS with mottled pigmentation subtype (EBS-MP; MIM 131960) (8). Mutation c.1649delG, located in the tail domain of K5, causes the EBS with migratory circinate erythema (EBS-MCE; MIM 609352), a singular clinical phenotype characterised by arcuate migratory inflammatory blistering lesions that heal with brownish pigmentation (9, 10). The pathogenic mechanism through which the c.1649delG mutation would cause the peculiar lesions of EBS-MCE remains elusive. Here, we report on an EBS-MCE patient and document the presence of immunocompetent cells in an active lesion of the patient, who bears the c.1649delG variant in *KRT5*. Our results provide evidence for an involvement of the cellular immunity in the expression of the disorder.

MATERIALS AND METHODS

Patient

The diagnosis of EBS was established during the neonatal period on the basis of the clinical features and confirmed by electron

 doi: 10.2340/00015555-1691
 ISSN 0001-5555

microscopy examination of a lesional skin biopsy. At the age of 3 years, a blood sample and a 4 mm punch biopsy from the edge of the migratory erythema were taken for genetic analysis and immunopathological studies, respectively. Biological samples were obtained after written informed consent of the parents. The study was conducted in compliance with the Declaration of Helsinki principles.

Mutation detection

Mutational analysis was performed by direct bidirectional sequencing of *KRT5* exons, as described (11). A second PCR reaction and resequencing of the PCR product was performed to verify the mutation further. Total RNA was extracted from the patient skin biopsy using TRIzol reagents (Invitrogen, Carlsbad, CA, USA). The cDNA was synthesised using Superscript III RT (Invitrogen), and amplified with *KRT5* intron-crossing primers that hybridise on exon 7 (5'-GGAGCTCATGAACACCAAGC) and exon 9 (5'-CTTGGAAGGCAGTGACTTGC).

Immunohistochemistry

The 4 mm punch biopsy, obtained at the age of 3 years from the active edge of a migrating lesion, was frozen at –80°C in OCT compound for immunofluorescence and immunohistochemical investigations and TUNEL staining. Immunofluorescence examination was performed on frozen 5-µm-thick sections using a 3-step biotin-streptavidin-fluorescein procedure, as described (12). K5 was probed with monoclonal antibody (mAb) C50 (Laboratory Vision, Fremont, CA, 1:50 dilution).

For immunohistochemistry, 5-µm cryostat sections were fixed in 4% paraformaldehyde for 5 min and pre-treated for 15 min with PBS containing 0.3% hydrogen peroxide to block the endogenous peroxidase activity, and with protein block serum-free solution (DAKO, Glostrup, Denmark) to block the nonspecific binding of primary and secondary Abs. Slides were incubated for 1 h at room temperature in a humid atmosphere with 2.5 µg/ml of anti-CD1a (HI149, mIgG1), 5 µg/ml of anti-CD3 (HIT3a, IgG2a), 2.5 µg/ml of anti-CD4 (RPA-T4, mIgG1), 1.2 µg/ml of anti-CD8 (RPA-T8, mIgG1), 50 µg/ml of anti-CD69 (FN50, mIgG1) and 2.5 µg/ml of anti-CD83 (HB15e, mIgG1) mAbs (all from BD Bioscience,San Diego, CA), followed by biotinylated anti-mouse (DAKO) secondary Ab for 10 min. Streptavidin-HRP complex (DAKO) was added for 10 min to the samples, and 3-amino-9-ethyl-carbazole chromogen (Vector Laboratories, Burlingame, CA) was used as substrate. Every step was followed by extensive washing in PBS. Slides were counterstained with haematoxylin. As a negative control, primary Abs were omitted or replaced with irrelevant isotype-matched Abs.

TUNEL staining

In Situ Cell Death Detection Kit (Roche Applied Science, Penzberg, Germany) was used to identify DNA strand breaks by labelling free 3'-OH termini with fluorescein nucleotides in a template independent manner enzymatic reaction. Briefly, cryopreserved tissue sections were fixed with 4% paraformaldehyde for 5 min, incubated with 0.3% H_2O_2 for 15 min to inactivate endogenous peroxidase and permeabilised with 0.05% Triton for 5 min at room temperature. Afterwards, sections were incubated with terminal deoxynucleotidyl transferase enzyme and fluorescein nucleotides mixture for 60 min at 37ºC in a humidified chamber in the dark. After 3 washes in PBS, the fluorescein labels incorporated in nucleotide polymers were detected and quantified by fluorescence microscopy using an excitation wavelength of 488 nm and a detection of 515–565 nm.

RESULTS

Clinical features and laboratory findings

The patient was a female infant, first child of non-consanguineous parents. Since the 4th day of life, she presented trauma-induced blisters at first localised to hands and feet (Fig. 1a) that rapidly spread to the entire body surface. Blistering tendency markedly decreased after the first year of life, when lesions continued to form only on hands and feet and in other body areas exposed to trauma. By the age of 2 years the patient presented a generalised reticulated pigmentation affecting in particular the neck, axillary and inguinal folds, and limbs (Fig. 1b). In addition, circinate and migrating areas of erythema with multiple vesicles and small crusts at the advancing edge developed on the nape and shoulders (Fig. 1c). The erythematous lesions were extremely itchy and resolved with a patchy hyperpigmentation. The hair and nails were normal and mucosae were not affected. At subsequent follow-up the disappearance of the migratory erythematous lesions was noticed by the age of 4 years following sun exposure in summer. No erythematous migrating lesions were noticed for the subsequent 6 years (Fig. 1d). At the age of 10 years the patient suddenly developed a widespread eruption of itchy erythematous papules and small plaques, often annular-shaped or circinate, and with occasional tiny vesicles in the periphery (Fig. 1e, f). Additional findings include residual mottled pigmentation, in particular of axillary folds (Fig. 1e), mild onychodystrophy, mainly of the toenails, and rare trauma-induced foot blisters. Family history revealed that the mother had suffered from acral blistering during the first year of life followed by development of a reticulated pigmentation that disappeared by the age of 18 years. The mother and grandmother did not remember any migrating erythematous lesion. The mother still presents minimal onychodystrophy (Fig. S1[1]).

Diagnosis of EBS-MCE in the index case was based on clinical examination and ultrastructural findings (not shown) in infancy. The latter showed cleavage within basal keratinocyte cytoplasm, in the absence of tonofilament clumping. Moreover, immunofluorescence analysis of the biopsy obtained at the age of 3 years revealed epidermal basal vacuolisation and an irregular labelling of basal keratinocytes for K5 (not shown). The clinical diagnosis was confirmed by *KRT5* sequencing, which revealed the c.1649delG mutation at the heterozygous state in the DNA of the proband and her mother (Fig. 2). The expression of the mutant allele was confirmed by direct sequencing of a cDNA fragment, which showed frame shifted sequences across the mutation site. However, the nucleotide peaks cor-

[1]http://www.medicaljournals.se/acta/content/?doi=10.2340/00015555-1691

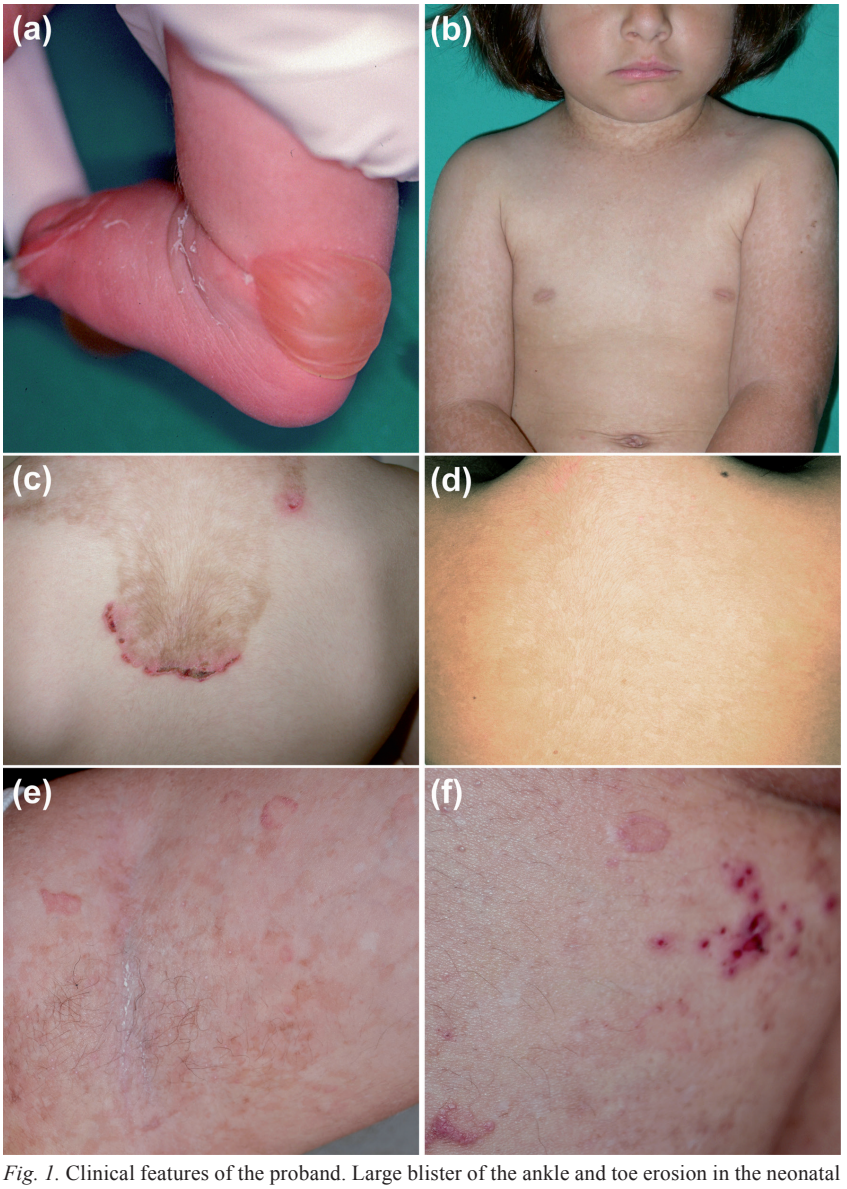

Fig. 1. Clinical features of the proband. Large blister of the ankle and toe erosion in the neonatal period (a). Diffuse mottled pigmentation, more evident on the upper limbs and neck (b), and migratory circinate erythematous vesicular and crusted lesions healing with patchy hyperpigmentation of the back (c) at the age of 3 years. Minimal residual reticulated pigmentation of the back, in the absence of any active lesion, at the age of 9.5 years (d). Annular erythematous small plaques and reticular hyperpigmentation of the axillary fold at the age of 10 years (e). Circinate and arcuate erythematous plaques and excoriated erythematous vesicles of the upper posterior thigh at the age of 10 years (f).

responding to the mutant cDNA were lower than the wild-type cDNA. This finding indicates that the mutant mRNA is less abundant than the normal one, possibly due to a reduced stability (Fig. 2, bottom panel).

Immunohistochemical studies and TUNEL assay

The characteristics of the inflammatory infiltrate at the active border of an erythematous lesion were investigated by immunohistochemistry. We found that the majority of the inflammatory cells are $CD3^+$ T lymphocytes with a slight prevalence of the $CD4^+$ T-cell subset over the $CD8^+$ subpopulation (Fig. 3a–c). Both $CD4^+$ and $CD8^+$ T cells were predominantly distributed in the upper dermis, but a substantial number of the infiltrating lymphocytes entered the epidermis in close contact with basal and suprabasal keratinocytes. Interestingly, the activation marker CD69 was expressed by more than 50% of infiltrating cells (Fig. 3d). The number of intraepidermal $CD1a^+$ Langerhans' cells was not increased (Fig. 3e). However, a high number of dendritic cells expressing the activation marker CD83 were present in the upper dermis (Fig. 3f). TUNEL staining revealed numerous apoptotic keratinocytes scattered throughout all epidermal layers in lesional skin (Fig. 3g).

DISCUSSION

The patient here reported confirms the tight phenotype-genotype correlation of the c.1649delG mutation in EBS-MCE (6, 9, 10). This deletion is predicted to cause a frame shift that alters the sequence of the last 41 amino acids and adds 35 residues to the tail of K5. However, mutation consequences at the mRNA and protein level have never been investigated *in vivo*. Our analysis on total RNA extracted from the patient skin biopsy showed a reduced amount of transcripts encoding for mutant K5 polypeptides. On the other hand, an *in vitro* study has demonstrated that the keratin filaments assembled from wild type K14 and mutant K5 lacking the last 41 residues are shortened and weakened in their viscoelastic properties when subjected to strain (13). These alterations can account for the skin fragility, but do not explain the inflammatory and migrating lesions. Moreover, the c.1649delG mutation has also been associated with EBS with mottled pigmentation (8, 14, 15). Our case shows features of both EBS-MCE, with typical lesions at first localised to the nape and back and later in a more widespread distribution, and EBS with mottled pigmentation, more evident on the limbs and large folds. In addition, the mother of the patient had a history compatible with EBS with mottled pigmentation alone. Therefore the c.1649delG mutation seems to result in the majority of patients in EBS with

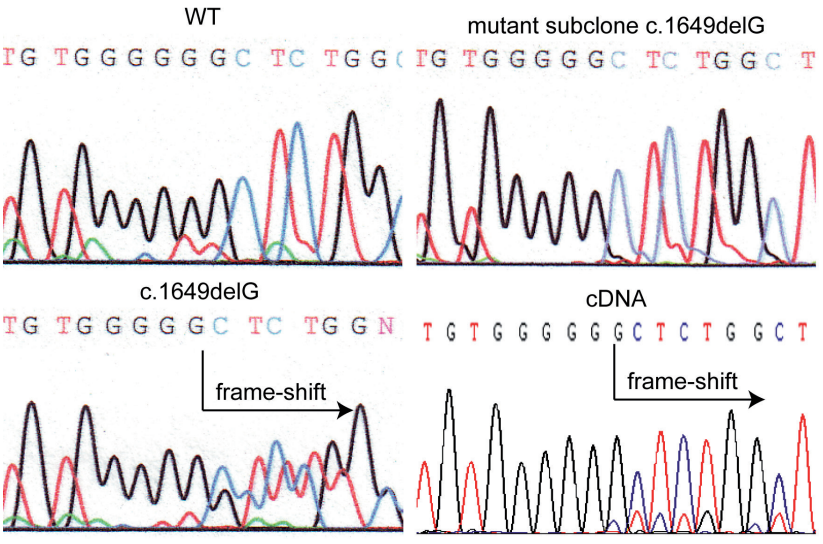

Fig. 2. Identification of the c.1649delG mutation in *KRT5* and mRNA analysis. Sequence chromatogram of exon 9 amplified from the index case reveals a heterozygous nucleotide deletion within a run of 6 consecutive guanosine. The deletion of a guanosine is evident in the sequence of a subcloned allele. WT, wild-type sequence of *KRT5* exon 9 from a normal control. Sequence analysis of a cDNA fragment encompassing the mutation site (bottom right panel). Note that the nucleotide peaks corresponding to the mutant cDNA are lower than the wild-type cDNA.

mottled pigmentation and in some cases to also cause MCE, implying that additional molecular modifiers are involved in MCE pathogenesis. The absence of EBS-MCE lesions in our patient during 6 years also suggests that environmental factors could be involved in triggering the inflammatory lesions in these patients.

The intervention of immune responses orchestrated by locally recruited dendritic cells that activate immune functions of skin T lymphocytes might partially explain the typical erythematous lesions occurring in MCE (16). Our results demonstrated the presence of a heavy leukocyte infiltrate at the edge of the advancing erythematous lesions. The presence of $CD3^+$, $CD4^+$ cells within the epidermis and, to a lesser extent, of $CD8^+$ T-cell subsets in the upper dermis, was ascertained (Fig. 3a–c). Many infiltrating cells in physical contact with

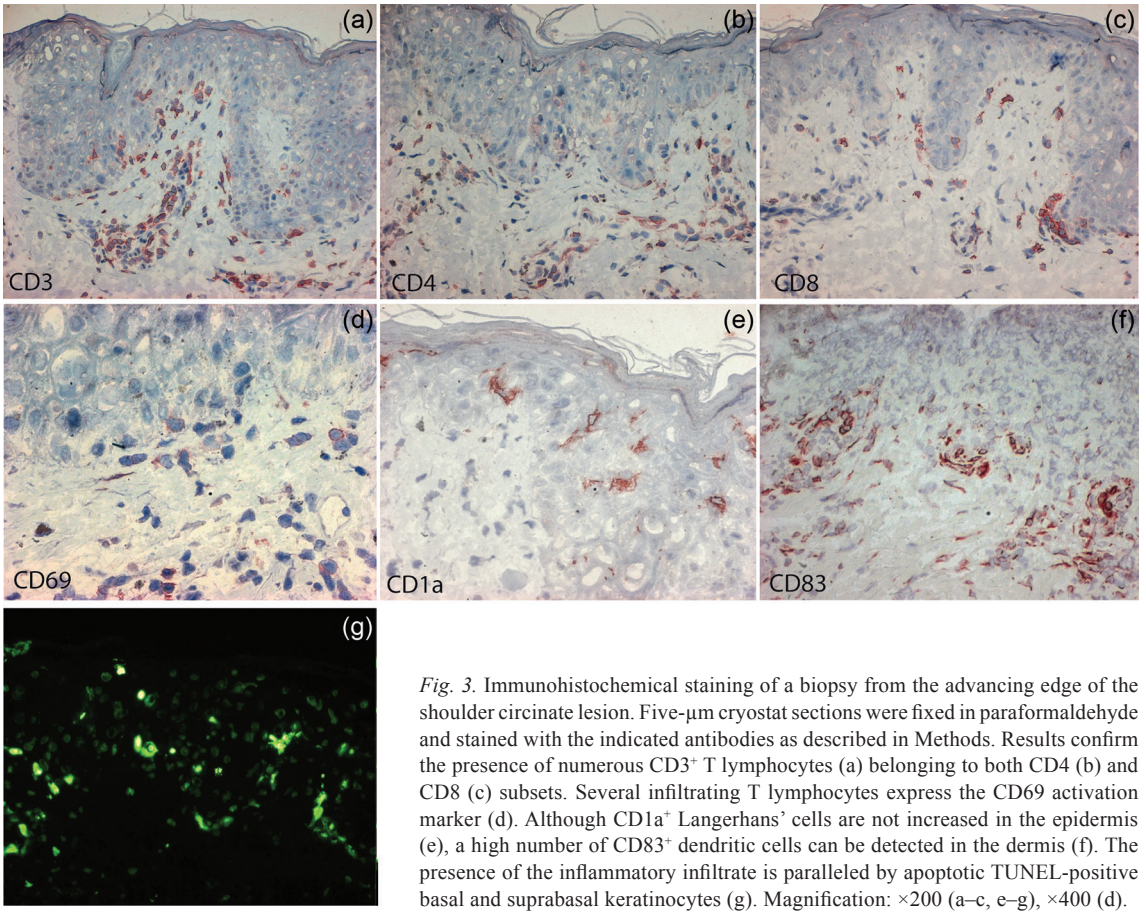

Fig. 3. Immunohistochemical staining of a biopsy from the advancing edge of the shoulder circinate lesion. Five-µm cryostat sections were fixed in paraformaldehyde and stained with the indicated antibodies as described in Methods. Results confirm the presence of numerous $CD3^+$ T lymphocytes (a) belonging to both CD4 (b) and CD8 (c) subsets. Several infiltrating T lymphocytes express the CD69 activation marker (d). Although $CD1a^+$ Langerhans' cells are not increased in the epidermis (e), a high number of $CD83^+$ dendritic cells can be detected in the dermis (f). The presence of the inflammatory infiltrate is paralleled by apoptotic TUNEL-positive basal and suprabasal keratinocytes (g). Magnification: ×200 (a–c, e–g), ×400 (d).

basal and suprabasal keratinocytes expressed the CD69 activation marker (Fig. 3d). Altogether, these findings support the hypothesis of a prominent role of the skin immune system in sustaining the clinical expression of the disease. Previous reports demonstrated the increase of the chemokines CCL2, CCL19 and CCL20 in the skin of keratin $5^{-/-}$, but not keratin $14^{-/-}$, mice (17, 18). CCL20 is a strong chemoattractant for T lymphocytes, Th17 cells in particular, and may thus be responsible for the accumulation of effector T lymphocytes in the skin, which could contribute to the keratinocyte damage. Indeed, the presence of apoptotic TUNEL-positive basal and suprabasal keratinocytes in lesional skin (Fig. 3g) suggests the role of perforin- and/or FAS/FasL-mediated T-cell cytotoxicity.

In line with the hypothesis of a prominent role of the immune system in the expression of the disease, MCE manifestations in our patient were cleared for several years after summer exposure to UV radiation, a known modulator of both innate and adaptive skin immune responses (Fig. 1).

Although at the advancing edge of the circinate inflammatory lesions we could not detect a significant increase of the number of intraepidermal Langerhans' cells (Fig. 3e), as previously described in keratin $5^{-/-}$ mouse and in patients affected with EBS by Roth et al. (17), our patient displayed a high number of dendritic cells in the upper dermis expressing the activation marker CD83 (Fig. 3f). This finding confirms the active recruitment and maturation of dendritic cells at the lesional site. Whether the inflammatory response depends on a specific immune response to neo-antigens released by damaged keratinocytes, or is the consequence of an unspecific response following the release of pro-inflammatory cytokines and chemokines in affected areas remains to be established.

ACKNOWLEDGMENT

This work was supported by grants from Italian Ministry of Health (RC11-1.2 and RC11-1.4).

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