Actas Dermosifiliogr. 2010;**101(8)**:673–682

ACTAS
Dermo-Sifiliográficas

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NOVELTIES IN DERMATOLOGY

Immunofluorescence Mapping for Diagnosis of Congenital Epidermolysis Bullosa

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Manuscript received March 11, 2010; accepted for publication June 10, 2010

KEYWORDS
Epidermolysis bullosa;
Classification;
Antigen mapping;
Immunofluorescence

Abstract
The tools for diagnosis of epidermolysis bullosa have advanced greatly since Hintner's group introduced antigen mapping as a diagnostic test for this family of genodermatoses. Monoclonal or polyclonal antibodies raised against some of the specific proteins found in the epidermis and basement membrane of the epidermis have allowed 4 types of epidermolysis bullosa de be identified and all variants to be classified. When a newborn baby presents with blisters, many conditions are implicated in the differential diagnosis. Examination under an optical microscope can suggest epidermolysis bullosa, but immunofluorescence mapping and electron microscopy are required for confirmation of the diagnosis and further classification of congenital epidermolysis bullosa. This article explains the importance of immunofluorescence antigen mapping and describes the methods employed for classification and subclassification of epidermolysis bullosa.

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PALABRAS CLAVE
Epidermólisis ampollosa;
Clasificación;
Mapeo antigénico;
Inmunofluorescencia

Mapeo por inmunofluorescencia para el diagnóstico de epidermólisis ampollosa congénita

Resumen
Las herramientas para el diagnóstico en las epidermólisis ampollosas (EA) han tenido un gran avance desde que Hintner et al introdujeron el mapeo antigénico como prueba diagnóstica en este grupo de genodermatosis. La utilización de anticuerpos monoclonales/policlonales dirigidos contra algunas de las proteínas específicas que conforman la epidermis y la membrana basal epidérmica han servido para clasificar los 4 tipos de epidermólisis ampollosa y subclasificar todas sus variantes. Ante la presencia de un recién nacido con ampollas surgen diagnósticos diferenciales múltiples, en donde la microscopia de luz orienta el diagnostico de epidermólisis ampollosa. Sin embargo, el mapeo por inmunofluorescencia y la microscopia electrónica permiten confirmar y clasificar a las epidermólisis ampollosas congénitas.
En este artículo, se explica la importancia y metodología para desarrollar la técnica de mapeo antigénico por inmunofluorescencia, con el propósito de clasificar y subclasificar las epidermólisis ampollosas.

Introduction

The term epidermolysis bullosa was first used by Koebner in 1886 to describe a group of clinically heterogeneous genetic diseases characterized by the appearance of blisters, erosions, and ulcers on the skin and/or mucosas, either spontaneously or after minor trauma.[1] In view of these characteristics, they are also known as mechanobullous disorders.

More than 30 subtypes of have been described according to phenotype, type of transmission, and genotype. However, 3 main types have been identified according to the structural level in the skin at which the blisters develop, as identified by immunofluorescence and/or electron microscopy; these types are epidermolysis bullosa simplex, junctional epidermolysis bullosa, and dystrophic epidermolysis bullosa.[2,3] In the simplex forms, the lesions are located in the epidermis (epidermolytic form) whereas in the junctional form, cleavage of the dermoepidermal junction can be clearly observed.[4,5] In the dystrophic forms, the lesions develop in the papillary dermis (dermolytic form). The new classification, published in 2008 and representing international consensus on the diagnosis of epidermolysis bullosa, introduced a fourth mixed type (Kindler syndrome) when the blisters of affected skin involve more than 1 structural level (Figure 1).[6]

The challenge of accurately diagnosing the type of epidermolysis bullosa is particularly difficult if based solely on clinical presentation, and even more so if the family history of the patient is not known.[7] A newborn baby will readily develop blisters and erosions in response to external agents such as heat, chemical irritants, and trauma (Figure 2).[8,9] Moreover, most of the diseases characterized by skin fragility will present during the neonatal period. The differential diagnoses for congenital epidermolysis bullosa therefore takes into account several disorders, from transient benign diseases through to life-threatening mutilating deformities. Differential diagnosis should include suction blisters, bullous congenital ichthyosiform erythroderma, bullous impetigo, staphylococcal scalded skin syndrome, neonatal herpes simplex, intrauterine varicella-zoster, incontinentia pigmenti (Bloch-Sulzberger syndrome), cutis aplasia, focal dermal hypoplasia (Goltz syndrome), congenital erythropoietic porphyria (Gunther disease),

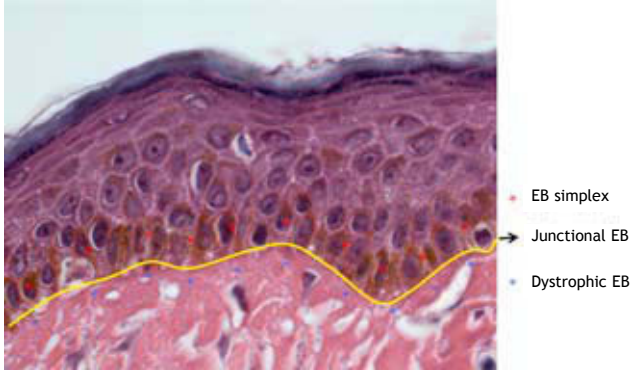

Figure 1 Epidermal basement membrane and location of main types of epidermolysis bullosa (EB). Hematoxylin and eosin (×100).

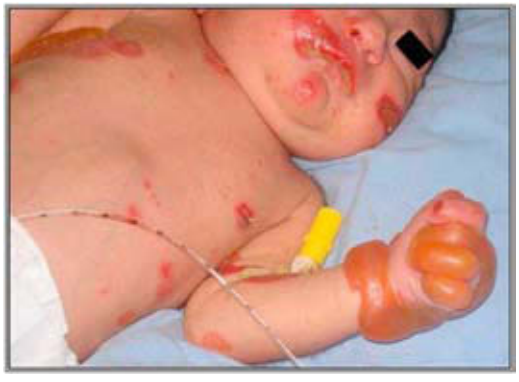

Figure 2 Newborn baby with disseminated blisters on the face, chest, and hands.

neonatal lupus erythematosus, ecthyma gangrenosum, *Aspergillus* infection, and immunobullous disorders such as pemphigus or pemphigoid gestionis.[8-11]

In infants and adults, congenital epidermolysis bullosa also exhibits clinical characteristics reminiscent of bullous mastocytosis, toxic epidermal necrolysis, and certain autoimmune diseases such as bullous pemphigoid, cicatricial pemphigoid, linear immunoglobulin A disease, and epidermolysis bullosa acquisita.[9-11]

Diagnosis of epidermolysis bullosa should be considered once these diseases have been ruled out by recording a detailed clinical history, performing a complete physical examination, culturing samples from the lesions with Gram and Giemsa stains (to rule out bacterial infections), taking Tzanck smears (when viral infection is suspected), or applying potassium hydroxide (to rule out mycosis). When epidermolysis bullosa is suspected, a skin biopsy is taken, stained with hematoxylin and eosin, and antigen mapping is performed using immunofluorescence techniques and/or the sample is examined under an electron microscope. The aim of immunofluorescence and electron microscopy is to determine the cleavage plane and whether the blisters form in the epidermis or epidermal basement membrane.[9-13]

Optical microscopy is not very useful for diagnosis of the disease given that it is only possible to visualize a subepidermal blister with few inflammatory cells, and little difference can be seen between the different types of epidermolysis bullosa. However, in some cases of epidermolysis bullosa simplex, intraepidermal blisters can be seen to form in the basal layer as a result of cytolysis of basal keratinocytes (Figure 3).[14]

The first laboratory technique successfully used to carry out a precise diagnosis and classification of the disease was electron microscopy. For many years this technique was the most widely used as it allowed the structure of the cells, organelles, cytoplasm (and the cytokeratin tonofilaments in particular), hemidesmosomes, and anchoring fibrils involved in this disease to be visualized in detail.[15] In addition, electron microscopy can reveal whether separation occurs at the level of the basal keratinocytes and/or basement membrane. However, this technique is expensive, time-consuming, and requires specialists with experience in the disease.

Thus, interpretation of an electron microscopy study can be, at times, imprecise and there are only a few laboratories with staff that have the appropriate experience and skill for analyzing and interpreting samples from patients with congenital epidermolysis bullosa.[5,7] In 1981, Hinter et al[5] were the first to describe the technique of immunofluorescence antigen mapping of this disease. Their technique is based on detection of structural proteins in the epidermis and/or dermoepidermal junction using polyclonal and/or monoclonal antibodies. The blister location and anchoring site is determined from the location of a given antigen (such as, for example, type IV collagen antigen) in a naturally-occurring or induced blister. Depending on the antibodies used, this technique confirms whether the expression of structural proteins is normal, reduced, or absent. Currently, several antibodies can recognize known pathological structural proteins of keratinocytes and/or of the dermoepidermal junction in the basement membrane.

Fortunately, some of these antibodies for carrying out cutaneous fluorescence are commercially available worldwide.[6,16,17] Immunofluorescence mapping has ousted electron microscopy and is currently the primary laboratory technique used for diagnosing epidermolysis bullosa and for differentiating between different subtypes. In addition, the technique forms the basis for determining the proteins to be targeted in the mutation analysis.[6,15]

Biopsy

In a patient with suspected congenital epidermolysis bullosa, the biopsy should preferably be taken from healthy skin around a recent blister (Figure 4). When there are no recent blisters, healthy skin should be rubbed with an eraser for 2 minutes to induce new blister formation.

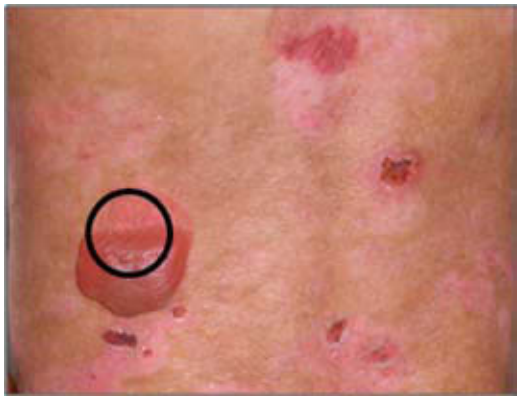

Figure 4 Ideal site for biopsy for immunofluorescence studies.

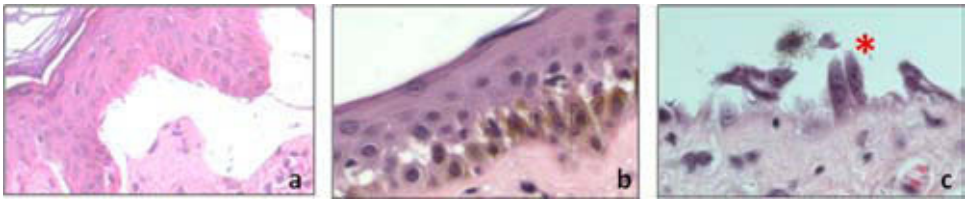

Figure 3 a) Subepidermal blister lacking inflammatory cells characteristic of any form of epidermolysis bullosa. Hematoxylin and eosin (×20). b) Basal blister characteristic of any form of epidermolysis bullosa simplex. Hematoxylin and eosin (×20). c), Two areas of keratinocyte remnants adhering to the basement membrane in epidermolysis bullosa simplex (*). Hematoxylin and eosin (×60).

The rationale for taking a biopsy from recently formed or induced blisters is that these will not exhibit changes in structural proteins caused by the wound itself or the healing process.[5-7,18]

Fixing and Storage of the Sample

The skin sample should be immediately placed in Michel solution, which was initially described by Michel in 1973 and modified by Vaughan in 1995; this solution consists of 2.5 mL of 1 M citrate buffer (pH 7.4), 5 mL of 0.1 M magnesium sulfate, 5 mL of 0.1 M N-ethylmaleimide, and 55 g of ammonium sulfate dissolved in 87.5 mL of water to yield a final volume of 100 mL, with the pH adjusted to 7.4 with 1 M sodium hydroxide.[18-20] Samples can be stored in this medium for 28 days at room temperature to allow shipment to a specialized laboratory anywhere in the world for antigen mapping. To improve diagnostic sensitivity, the sample should be washed in phosphate buffered saline (PBS) for several hours before sectioning, which should be done in a cryostat.[21-23] The samples will then be ready for staining.

Antibodies

The primary antibodies for cutaneous immunofluorescent mapping are derived from various animals (rat, mouse, rabbit) and bind to specific target structural proteins in the skin. Antibodies that can be used for antigen mapping are raised against cytokeratin 5, cytokeratin 14, plectin, integrin $\alpha 6$ and $\beta 4$, type XVII collagen (180-kD bullous pemphigoid antigen 2), laminin 332 (formerly known as laminin 5) with its 3 chains ($\alpha 3$, $\beta 3$, and $\gamma 2$), and finally type VII collagen.[24,25] To improve the visualization of the blister, particularly in patients with dystrophic epidermolysis bullosa, antibodies raised against type IV collagen (present in the lamina densa of the dermoepidermal junction) are used (Table 1).

All of the previously mentioned antibodies are of the immunoglobulin (Ig) G type; most of them are monoclonal and developed in mice. Therefore, the secondary antibody should be raised against the IgG antibody, normally coupled to a fluorescein isothiocyanate group. An exception is the antibody against integrin $\alpha 6$, which is raised in rat, and so the secondary antibody is of the anti-rat IgG type. The fluorescein isothiocyanate group binds to the secondary antibody and fluoresces over a wavelength range of 450-490 nm, allowing detection of the specific protein-bound antibody by fluorescence microscopy.

The quantities of antibodies stored will depend on how often they are needed for immunofluorescence and the final dilution.[22] There should be enough antibody on hand to allow for fresh preparation of all samples to be analyzed in each session. The antibodies are stored frozen until their expiry date to avoid contamination.

Staining Technique

During the staining for immunofluorescence mapping, it is recommended to use 3 to 4 sections for each sample, each one 4 to 6 µm thick, as well as 1 or 2 skin sections from healthy patients as positive controls (Figure 5). This approach provides a reference for immunofluorescence of the target structural proteins for subsequent comparison of the immunoreactivity of proteins from healthy controls with that of proteins from patients with epidermolysis bullosa.

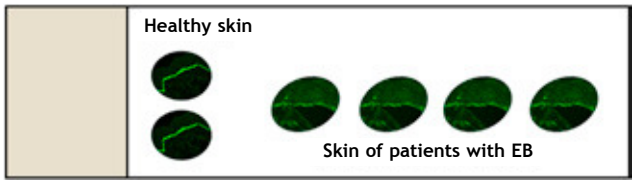

Figure 5 Preparation of a slide for immunofluorescence staining. EB indicates epidermolysis bullosa.

Table 1 List of Antibodies and Dilutions Currently Used in Immunofluorescence Studies to Diagnose Epidermolysis Bullosa

	Supplier	Dilution	Host	EB Subtype
Primary Antibody				
Cytokeratin 5	Millipore	1:50	Mouse	EBS
Cytokeratin 14	Millipore	1:100	Mouse	EBS
Collagen XVII	Marinkovich	1:20	Mouse	JEB
Plectin	Wiche	1:2	Mouse	EBS, JEB
Integrin α-6	Millipore	1:50	Rat	EBS, JEB
Integrin β-4	Millipore	1:50	Mouse	EBS, JEB
Laminin 332[a]	Millipore	1:50	Mouse	JEB
Collagen IV	Millipore	1:50	Mouse	—
Collagen VII	Millipore	1:50	Mouse	DEB
Secondary Antibody				
IgG	Millipore	1:100-400	Anti-rat	
IgG	Dako	1:100	Anti-mouse	

[a]Formerly laminin 5.

The number of slides prepared will correspond to the number of antibodies for staining of the possible target proteins. Two will be used as negative controls to assess and account for nonspecific immunoreactivity of the secondary antibody. The slides are incubated in PBS with the primary antibody, then incubated either with the secondary antibody coupled to fluorescein isothiocyanate or the anti-rat secondary antibody. Each slide is incubated with the primary antibody at the appropriate dilution (or PBS in the case of negative control) for 30 minutes in a moist chamber at room temperature. The samples are then washed twice with PBS and incubated with the secondary antibody at the appropriate dilution for 30 minutes in the same conditions.

Subsequently, the samples should be washed again twice with PBS for 15 minutes then covered immediately with glycerol and a coverslip. Finally, in the fluorescence microscopy, photographs of standard and fluorescence reactivity are taken and filed with the patient records. The samples for this technique remain stable for several weeks when kept refrigerated at 4°C.

Staining Patterns

With antigen immunofluorescence mapping, it is possible to visualize the location and expression of structural proteins involved in the pathogenesis of the different forms of epidermolysis bullosa, as well as the site of separation and/or blister formation. However, the intensity of protein staining is influenced by several factors such as the part of the body from which the skin was taken (normal or blistered skin), prior exposure to sunlight, and the patient's age. To ensure a suitable correlation between diseased skin samples and healthy control skin, samples should be taken from the same part of the body and patients and controls should be age-matched. In addition, the duration of storage in Michel solution (preferably less than 28 days), shipping conditions (high temperatures, freeze-thawing), and storage conditions of the antibodies can affect the results of immunofluorescence. To evaluate the stained samples, it is useful to consider a scale of 1 to 4 according to the intensity of the antigen-antibody reaction, where (+) is doubtful fluorescence, (++) weak fluorescence, (+++) moderate fluorescence, and (++++) strong fluorescence. As mentioned earlier, it is of utmost importance that the biopsy includes tissue from the blister to determine the structural level at which separation occurs.

In the case of epidermolysis bullosa simplex, when, for example, the collagen IV antibody (present in the lamina densa of the dermoepidermal junction) is used, intraepidermal blisters are found in the basal keratinocyte layer. When this occurs, we see that the fluorescence, emanating from the lamina densa, remains at the base of the blister alongside the remnants of basal keratinocytes bound to the basement membrane (Figure 6a).

When studying junctional epidermolysis bullosa lesions, antibodies against collagen IV and laminins are usually used for the immunofluorescence techniques. In such cases, fluorescence is located at the base of the blister (Figure 6b and 6c), although in the case of cytokeratins, labeling only occurs at the roof of the blister (Figure 6b).

Finally, in cases of dystrophic epidermolysis bullosa, mapping studies are usually done with antibodies against

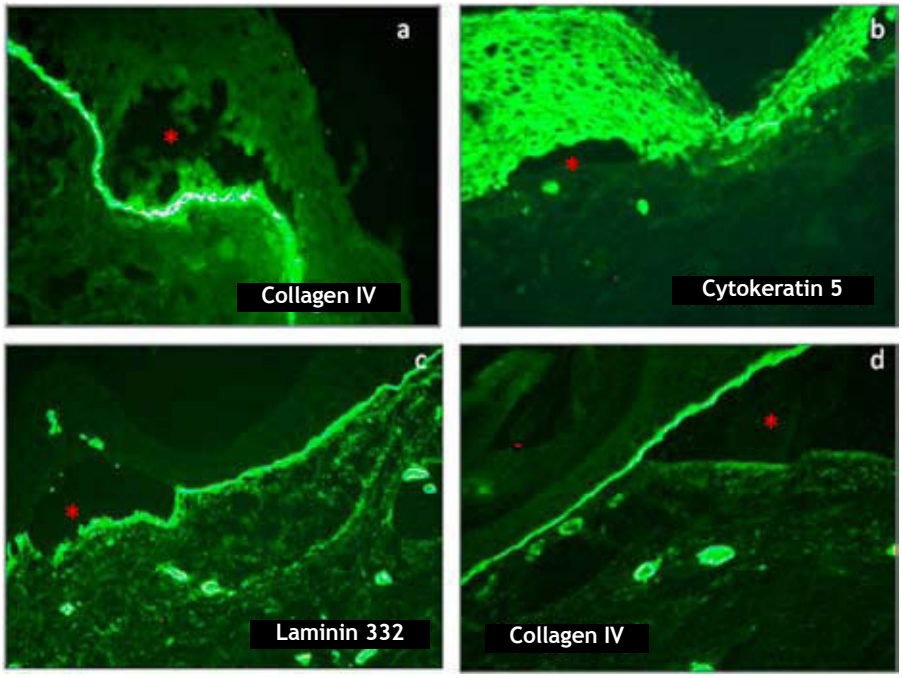

Figure 6 a) Epidermal blister associated with cytokeratin 5 and 14, plectin and integrins $\alpha 6\beta 4$ (×20). b and c) Blister in the lamina lucida (×20) (cytokeratin 5 and lamina 332). d) Blister in the papillary dermis below the lamina lucida (×20) (collagen VII). The asterisk (*) indicates blisters.

Table 2 Abnormal Antigens in Epidermolysis Bullosa

Type	Major Subtype	Subtype	Targeted Protein	Blister Site	Staining Pattern
Epidermolysis bullosa simplex (EBS)	Suprabasal	*Lethal acantholytic*	Desmoplakin	Suprabasal	
		Plakophilin deficiency	Plakophilin 1	Suprabasal	
		EBS superficialis	?	Suprabasal	
	Basal	Localized EBS	Cytokeratins 5 and 14	Basal	Normal
		EBS, Downling-Meara type	Cytokeratins 5 and 14	Basal	Normal
		EBS, other generalized	Cytokeratins 5 and 14	Basal	Normal
		EBS with muscular dystrophy	Plectin	Basal (above the hemidesmosomes)	Absent or reduced
		EBS with mottled pigmentation	Cytokeratin 5	Basal	Normal
		EBS with pyloric atresia	Plectin	Basal (above the hemidesmosomes)	Absent or reduced
			Integrins $\alpha6\beta4$	Upper lamina lucida (transmembrane)	Absent or reduced
		EBS, autosomal recessive	Cytokeratin 14	Basal	Absent or highly reduced
		EBS, Ogna	Plectin	Basal (above the hemidesmosomes)	Reduced
		EBS, migratory circinate	Cytokeratin 5	Basal	Normal
Junctional epidermolysis bullosa (JEB)	JEB, Herlitz	—	Laminin-332	Lamina lucida (anchoring filaments)	Absent or highly reduced
	JEB, others	*JEB, generalized non-Herlitz*	Mainly laminin-332	Lamina lucida (anchoring filaments)	Reduced
			Type XVII collagen	Below the hemidesmosomes	Absent
		JEB, localized non-Herlitz	Type XVII collagen	Below the hemidesmosomes	Reduced
		JEB with pyloric atresia	Integrins $\alpha6\beta4$	Upper lamina lucida (transmembrane)	Reduced
		JEB, inversa	Laminin-332	Laminin lucida (anchoring filaments)	Reduced
		JEB, late onset	?	?	
		Laryngo-onycho-cutaneous syndrome	Laminin-332/$\alpha3$ chain	Laminin lucida (anchoring filaments)	
Dystrophic epidermolysis bullosa (DEB)	Dominant DEB (DDEB)	DDEB, generalized	Type VII collagen	Anchoring filaments of the sublamina densa	Normal
		DDEB, acral			
		DDEB, pretibial			
		DDEB, pruriginosa			
		DDEB, nails only			
		DDEB, bullous dermolysis of newborn			Granular pattern with basal and suprabasal keratinocytes. Absent

				or highly reduced. (Only during periods of active blistering)
Recessive dystrophic epidermolysis bullosa (RDEB)	RDEB, severe generalized	Type VII collagen	Anchoring filaments of the sublamina densa	Absent or highly reduced
	RDEB, generalized other			Reduced
	RDEB, inversa			Variable
	RDEB, pretibial			
	RDEB, pruriginosa			Normal
	RDEB, centripetalis			
	RDEB, bullosa dermolysis of newborn			

Sources: Fine JD et al,[6] Yiasemides et al.[7]

cytokeratins, collagen IV, and laminin antigens. Thus, fluorescence is located at the roof of the blister (Figure 6d).[26] For each type and subtype of the disease, different abnormal proteins can be detected in the skin. Expression of these proteins relative to controls may be the same, reduced, or completely absent (Table 2).

Epidermolysis Bullosa Simplex

In patients with epidermolysis bullosa simplex (Figure 7a), the main proteins involved in the pathogenesis of the disease are cytokeratins 5 and 14, plectin, integrin $\alpha 6$, and integrin $\beta 4$. Changes in some of these proteins favors the formation of intraepidermal blisters through cytolysis of basal keratinocytes (Figure 7b, 7c, and 7d). In general, the proteins are expressed to a similar extent to controls, except in the case of recessive autosomal epidermolysis bullosa simplex,[26] in which patients lack cytokeratin 14.

In patients with epidermolysis bullosa simplex with muscular dystrophy, plectin is generally absent and in rare cases such as epidermolysis bullosa simplex of Ogna, plectin is markedly reduced. In patients with pyloric atresia (epidermolysis bullosa simplex or junctional epidermolysis bullosa), plectin as well as integrins $\alpha 6\beta 4$ are reduced or absent.[27,28]

Junctional Epidermolysis Bullosa

The main target proteins in junctional epidermolysis bullosa are type XVII collagen (PBAg2) and laminin 332. Expression of these proteins can be normal, reduced, or absent compared to healthy controls. In cases with blisters in the dermoepidermal junction, immunoreactivity appears at the roof or the base of the blisters depending on the type of junctional epidermolysis bullosa (Figure 8).[29,30]

In cases of the Herlitz subtype, laminin 332 is absent or markedly reduced in most cases (Figure 8f). In cases of the generalized non-Herlitz subtype (Figure 8a-d), the affected proteins are laminin 332 or type XVII collagen. In most cases of the non-Herlitz subtype, laminin 332 is markedly reduced (Figure 8g), while in other patients, expression of type XVII collagen is absent or reduced. In patients who were previously classified as having generalized atrophic benign epidermolysis bullosa, the reactivity of laminin is similar to that of controls.

Dystrophic Epidermolysis Bullosa

The subtypes of dystrophic epidermolysis bullosa arise from mutations in the gene encoding type VII collagen. In patients with severe generalized epidermolysis bullosa (the recessive dystrophic form), type VII collagen immunofluorescence is absent in most cases (Figure 9a-d) and the antibody against type IV collagen is used to visualize the level of the blister. A reaction of the antibody against type IV collagen at the roof of the separation indicates the presence of a blister in the papillary dermis and confirms the dystrophic subtype of epidermolysis bullosa. On the other hand, when type IV

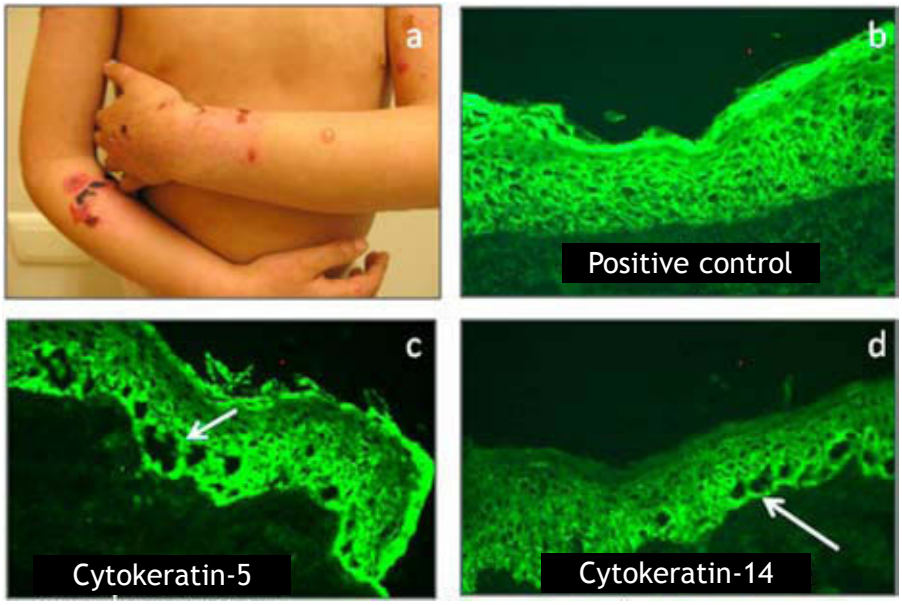

Figure 7 a) Clinical manifestations in patients with epidermolysis bullosa simplex. b) Positive control of healthy skin stained for cytokeratin 5 (×20). c and d) Staining for cytokeratin 5 and 14 to reveal basal intraepidermal blisters (×20).

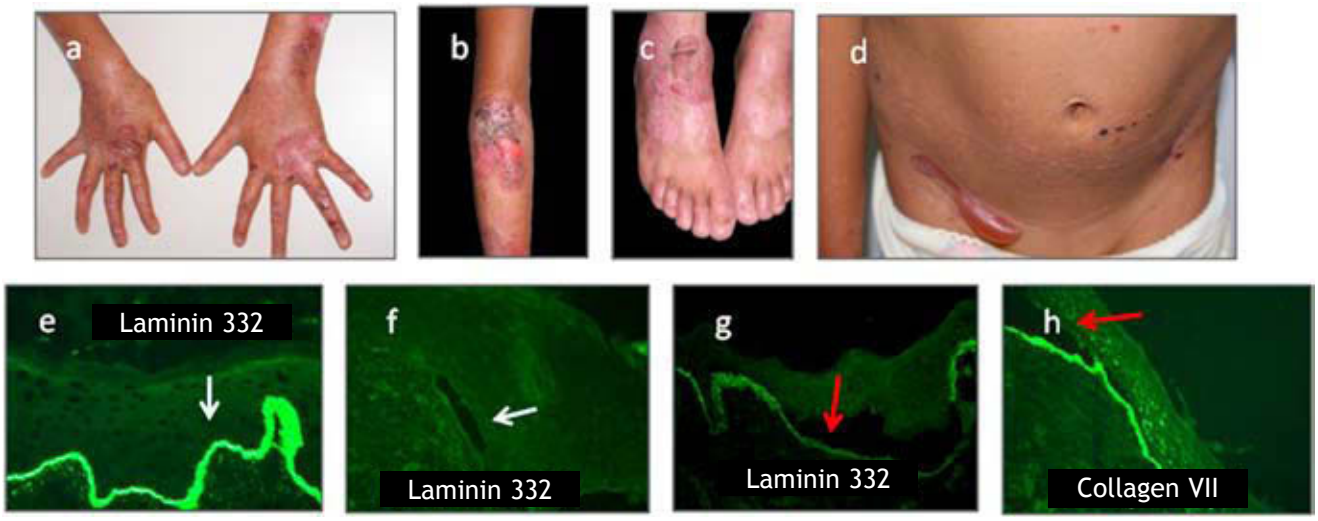

Figure 8 a-d) Patients with generalized non-Herlitz junctional epidermolysis bullosa (JEB); hemorrhagic blisters, onychodystrophy, tense blisters, and hemorrhagic crusts. e) Control laminin-332 (×20). f) Laminin-332 absent, characteristic of Herlitz JEB (×20). g) Laminin 5 of reduced intensity (×20). h) Type VII collagen (×20) of normal intensity in the roof of the blister (red arrows) characteristic of generalized non-Herlitz JEB.

collagen is detected at the base of the blister, a diagnosis of junctional epidermolysis bullosa or epidermolysis bullosa simplex subtypes should be considered (Figures 7 and 8).

Expression of type VII collagen may be reduced or even normal in other generalized subtypes of recessive dystrophic epidermolysis bullosa (Figure 9e-h).[6]

Conclusion

Antigen fluorescence mapping is the technique of choice for preliminary diagnosis and subsequent classification of the different types of epidermolysis bullosa. Patterns of immunofluorescence (to determine the depth of formation of blisters and visualize the expression of specific proteins) identify the proteins involved in the pathophysiology of the disease. Although the presence of the mutated genes in each patient is the defining criterion for diagnosing the disease, antigen mapping can assess expression of the epidermal proteins from the outset and thus locate the structural level of the epidermis affected. Subsequently, molecular studies can be used to analyze mutations in the genes that encode the affected proteins and so provide a definitive diagnosis.

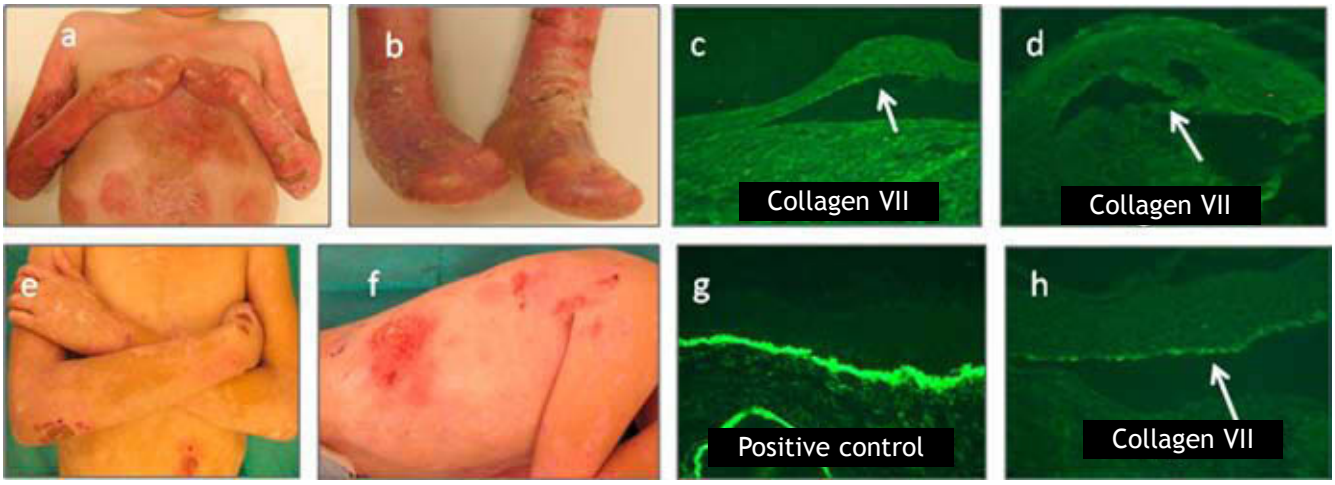

Figure 9 a and b) Severe generalized recessive dystrophic epidermolysis bullosa (RDEB) with complete pseudosyndactyly of both hands and feet accompanied by contraction. c and d) Complete absence of type VII collagen (×20). e and f) Generalized other RDEB with blisters, erosions, and atrophic scars on the chest and arms. g) Normal control (×20) for comparison with H. h) A blister in the papillary dermis with markedly reduced staining for type VII collagen (×20).

Immunofluorescence is the first useful laboratory technique to provide guidance to parents and patients concerning clinical prognosis, phenotype, and natural history of this disease, with the advantage that the results can be obtained the same day as the biopsy. In addition, this technique can be performed more easily in hospitals as the technical know-how required is not as demanding as it is for a technique such as electron microscopy. The availability of fixing media (such as Michel solution) that also allow shipment to laboratories specialized in epidermolysis bullosa may help physicians who make the initial diagnosis of this disease.

Funding

The present study was funded by the Hematology and Cancer Chair of the Center for Clinical Research, Monterrey Institute of Technology and Higher Education, Pathology Department, Hospital Universitario Dr. José E. González, Universidad Autónoma de Nuevo León, Mexico, DEBRA MEXICO AC, DEBRA AUSTRIA, and DEBRA INTERNATIONAL.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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