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De Novo Anti-Type VII Collagen Antibodies in Patients With Recessive Dystrophic Epidermolysis Bullosa

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The two main layers of human skin are held together by structures at the dermal-epidermal junction (DEJ) called anchoring fibrils (AFs). Without properly functioning AFs, the adherence between the epidermis and dermis is compromised. Clinically, this translates into skin fragility and skin bullae. AFs are composed of type VII collagen (C7) that has a central triple helical domain (TH) flanked by a 145-kDa non-collagenous amino-terminal domain (NC1) and a 30-kDa carboxyl-terminal domain (NC2) (Burgeson *et al*., 1993). AFs and C7 are perturbed in recessive dystrophic epidermolysis bullosa (RDEB), a disease characterized clinically by skin fragility, skin bullae, scarring, and nail loss (Fine *et al.*, 2008). RDEB is caused by mutations in the *COL7A1* gene encoding C7. Over 700 mutations have been identified in DEB patients (Wertheim -Tysarowska *et al.*, 2012). According to a recent consensus report, RDEB is classified as RDEB, severe, generalized (RDEB-sev, gen), RDEB, generalized, other (RDEB-O) and RDEB inversa (RDEB-I) (Fine *et al*., 2008).

There is also an acquired type of EB called epidermolysis bullosa acquisita (EBA). EBA patients are born with normal skin and then during middle age, they inappropriately generate IgG antibodies against their C7 and AFs (Yaoita *et al*., 1981, Woodley *et al*., 1984;) leading to skin fragility, trauma-induced blisters and scarring reminiscent of hereditary RDEB. The conventional wisdom in Dermatology is that patients with genetic RDEB may have a clinical phenotype resembling EBA, but that they have no auto-antibodies against C7. In this study, we identified 22 patients with *bona fide* RDEB, and characterized their mutations and their disease phenotype clinically, pathologically, ultrastructurally and immunologically. We sought to determine if any of these RDEB patients had anti-C7 antibodies in their sera or skin.

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Conflict of interest: Dr. Mei Chen, Dr. David T. Woodley and the University of Southern California hold patents for recombinant type VII collagen which are licensed by Shire Human Genetic Therapies. Drs. Chen and Woodley have filed a Conflict of Interest Declaration with Dr. Randoph W. Hall, Vice Provost for Research Advancement at the University of Southern California.

As summarized in Table I, 13 of the patients were classified as RDEB-sev, gen (patients 1–13) with *COL7A1* mutations that created premature termination codons (PTCs) due to nonsense or splice-site mutations (Spl), small insertions or deletions. Another nine RDEB patients (patients 14–22) had missense mutations (Mis) in one allele of *COL7A1* predicting glycine or arginine substitutions in the TH domain. Six patients (patients 14–19) had mutations associated with RDEB-I. Three patients had RDEB-O (patients 20–22). Of the 22 sequenced RDEB patients, 32 mutant alleles were identified. Nearly one third (10 of 32) of these mutations have not been previously reported.

We assessed the level of C7 expression at the DEJ of their skin by immunofluorescence staining of peri-lesional skin with a rabbit-anti-NC1 antibody (Chen *et al.*, 1997). As summarized in Table 1 and Supplementary on-line Figure S1, nine patients (patients 14–22) expressed C7 at the same level as skin from normal human subjects. The other RDEB patients had reduced (patients 1, 4–7, 9, 10, 12, 13) or no expression of C7 (patients 2, 3, 8, 11).

AFs were evaluated by transmission electron microscopy for density and morphology. As summarized in Table 2 and Supplementary on-line Figure S2, RDEB patients had reduced density or complete absence of AFs. When AFs were observed, they appeared attenuated in size or had an abnormal morphology.

To determine if RDEB patients have anti-C7 antibodies, we subjected our RDEB patients' sera to two different anti-C7 antibody ELISAs and immunoblot analysis. One commercially-available ELISA utilizes NC1 and NC 2 domains as the target substrate. The second ELISA is one we developed and employs full -length C7 as the target substrate. We used 13 EBA sera as positive controls and sera from 17 normal subjects as negative controls to establish the assay. The ELISA results are shown in Supplementary on-line Figures 3S and 4S and summarized in Table 2. With the commercial ELISA, 7 of 22 RDEB patient sera (patients 5, 6, 8, 9, 18, 20, 21) showed reactivity with values above the threshold. Similarly, in the full-length C7 ELISA, 11 of 22 patients exhibited reactivity. Using the full-length C7 ELISA allowed us to identify sera from four RDEB patients (patients 12, 16, 19, 22) that exclusively recognized the TH domain. These sera were further analyzed by immunoblotting against purified C7 (Woodley *et al.*, 2004). As summarized in Table 2 and Supplementary on-line Figures 5S, there is 100% correlation between ELISA and immunoblot results.

To determine if RDEB sera recognize C7 in the skin, we performed indirect immunofluorescence staining using salt-split human skin as substrate (Woodley *et al.*, 1984). None of the sera from these 11 patients bound to C7 on the dermal side of salt-split skin (data not shown). In addition, direct immunofluorescence of the 11 patients' skin did not detect any anti-C7 antibody deposits (data not shown), suggesting that the anti-C7 antibodies in their sera are likely non-pathogenic.

This study provides evidence that 12 of 22 *bona fide* RDEB patients have low level circulating anti-C7 autoantibodies that do not bind to the patients' skin. A previous smaller study found that 1 of 7 RDEB patients exhibited anti-C7 antibodies by ELISA (Pendaries *et al.*, 2010). In accordance with our data herein, a recent study of 17 RDEB patients showed

that 15 of 17 of the patients exhibited anti-C7 antibodies (Tampolini *et al.*, 2013). DIF on the RDEB patients, however, was not performed in either of these two studies.

Although our RDEB patients had varying types of *COL7A1* mutations, the expression of C7 in the DEJ of their skin ranged from none to the same as normal skin. The generation of anti-C7 antibodies is our RDEB cohort did not correlate with the expression of C7 in the patients' skin, the type of *COL7A1* mutation, the patients' age or the classification of RDEB. It is interesting to note that a correlation between anti-C7 antibodies and the Birmingham EB severity score was observed (Tampolini *et al.*, 2013).

All therapies for RDEB including cell therapy, protein therapy and vector therapy will involve exposure of the patient to new domains of C7 and the potential to generate anti-C7 autoantibodies (Chen *et al*., 2002, 2004, Wong e*t al*., 2008, Wagner e*t al*., 2010). The presence of anti-C7 antibodies in some RDEB patients prior to treatment should be taken into consideration when selecting and evaluating patients involved in clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

AFs	anchoring fibrils
CMP	cartilage matrix protein
DEJ	dermal-epidermal junction
C7	type VII collagen
EBA	epidermolysis bullosa acquisita
ELISA	enzyme-linked immunoabsorbant assay
IIF	indirect immunofluorescence
DIF	direct immunofluorescence
Fn3	fibronectin type III-like repeat
PTC	premature termination codon
RDEB	recessive dystrophic epidermolysis bullosa
NC1	N-terminal noncollagenous domain of type VII collagen
NC2	C-terminal noncollagenous domain of type VII collagen
RDEB-sev	gen, RDEB severe generalized

RDEB-O	RDEB generalized other
RDEB-I	RDEB inversa
TH	triple helical
VWF-A	A domain of von Willebrand factor

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Table 1

Summary of the clinical and mutational analysis of RDEB patients.

Patient ID	Patient Age	Allele 1 / Allele 2	Mutation Location	Consequences	Clinical Diagnosis
1	24	**G2517KfsX3 / G2517KfsX3**	TH / TH	PTC / PTC	RDEB-sev,gen
2	6	**c.356_357delCA/ c.356_357delCA**	CMP / CMP	PTC / PTC	RDEB-sev,gen
3	10	**c.356_357delCA / c.356_357delCA**	CMP / CMP	PTC / PTC	RDEB-sev,gen
4	27	c.4172dupC / **c.4182-4188dup7**	TH / TH	PTC/ PTC	RDEB-sev,gen
5	25	c.5048-5051dup4 / c.6501G-A	TH / TH	PTC / In-frame Del	RDEB-sev,gen
6	24	c.2993-5_3007dup20 / IVS64+4A>G	Fn3 / TH	PTC / Spl	RDEB-sev,gen
7	36	c.2993-5_3007dup20 / IVS64+4A>G	Fn3 / TH	PTC / Spl	RDEB-sev,gen
8	11	R578X / R578X	Fn3 / Fn3	PTC / PTC	RDEB-sev,gen
9	5	**P1523HfsX187 / IVS85-1G>T**	TH / TH	PTC / Spl	RDEB-sev,gen
10	3	R613X / R1683X	Fn3 / TH	PTC/ PTC	RDEB-sev,gen
11	34	c.7787delG / c.7787delG	TH / TH	PTC / PTC	RDEB-sev,gen
12	27	**IVS17-2delA/** R2814X	Fn3 / Acidic	Spl / PTC	RDEB-sev,gen
13	22	R236X /**IVS85-1G>A**	Fn3 / TH	PTC / Spl	RDEB-sen,gen
14	37	R2069C / 6501 G-A	TH / TH	Mis / In-frame Del	RDEB-I
15	23	R578X / G1907D	Fn3 / TH	PTC / Mis	RDEB-I
16	28	**IVS66+1 G**>C / G2719A	TH / TH	PTC / Mis	RDEB-I
17	62	R2069C / IVS5+1G>A	TH / CMP	Mis / PTC	RDEB-I
18	11	G1907D / c.6311_6312delCT	TH / TH	Mis / PTC	RDEB-I
19	38	G1907D / R1933X	TH / TH	Mis / PTC	RDEB-I
20	4	c.4919delG / G2366V	TH / TH	PTC / Mis	RDEB-O
21	45	**c.3582-3583delAG** / G1782R	VWA / TH	PTC / Mis	RDEB-O
22	31	G2233S / **IVS64-2_-1delAG**	TH / TH	Mis / Spl	RDEB-O

Abbreviations: TH, triple helical domain; CMP, cartilage matrix protein; VWA, A domain of von Willebrand factor (VWF-A); Fn3, fibronectin type III-like repeats; PTC, premature termination codon; Spl, splicing, Mis, missense; RDEB-sev, gen, RDEB, severe, generalized (formally Hallopeau-Simens RDEB); RDEB-O, RDEB, generalized, other (formerly Non-Hallopeau-Simens RDEB); RDEB-I, inversa type of RDEB. Newly identified mutations are bolded.

Table 2

Summary of C7 expression and AFs in RDEB patients' skin and anti-C7 antibodies in the blood.

Patient ID	C7 Expression at DEJ	Anchoring Fibrils by EM		NC1/NC2 ELISA	C7 ELISA	C7 Western Blot	Epitope
		Density	Morphology				
1	Reduced	+	Very thin and wispy	+/−	+	+	NC1/NC2
2	Absent	0	Absent	−	−	−	−
3	Absent	0	Absent	−	−	−	−
4	Reduced	0	Absent	−	−	−	−
5	Reduced	++	Thin, rarely arching	+	+	+	NC1/NC2
6	Reduced	+++	Thin, rarely arching	+	+	+	NC1/NC2
7	Reduced	++	Thin, rarely banded, rarely arching	−	−	−	−
8	Absent	+	Short, rudimentary	+	+	+	NC1/NC2
9	Reduced	++	Straight, non-banded	+	+	+	NC1/NC2
10	Reduced	+	Thin, mild arching	−	−	−	−
11	Absent	+	Short, rudimentary	−	−	−	−
12	Reduced	+	Thin and wispy	−	+	+	TH
13	Reduced	+	Thin and wispy	−	−	−	−
14	Normal	++++	Few banded, arching, looped	−	−	−	−
15	Normal	+++	Non-banded, arching	−	−	−	−
16	Normal	+++++	Banded, arching	−	+	+	TH
17	Normal	++++	Thin, arching, looped	−	−	−	−
18	Normal	+++	Non-banded, some arching	+	−	+	NC1/NC2
19	Normal	++++	Banded, arching	−	+	+	TH
20	Normal	+	Very thin and straight	+	+	+	NC1/NC2
21	Normal	++++	Thin and wispy, occasionally mild arching	+	+	+	NC1/NC2
22	Normal	+++	Thin, wispy, occasional arching	−	+	+	TH
NHS	Normal	+++++	Thick, banded, arching, looping	−	−	−	−
EBA	−	−	−	+	+	+	NC1/NC2

C7 expression at the DEJ was determined by immunofluorescence staining of cyrosections with an anti-NC1 antibody. AFs were evaluated by transmission EM, with the density indicated (0 indicates that no AFs were identified; five stars indicates normal density). The morphology of the individual AFs is qualitatively accessed from worst to best: absent, short or rudimentary, thin or wispy, arching, looping,

banded, thick. Normal individuals have a 5 star density with thick, banded, arching, and looping AFs. ELISA was performed with either a commercially available MBL kit that uses a mixture of immobilized NC1 and NC 2 domains as the target substrate or our recently developed assay that uses full-length, recombinant human *C7* as the target substrate. Immunoblot analysis was performed using purified recombinant *C7*.