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.
As summarized in Table I, 13 of the patients were classified as RDEB-sev, gen (patients 1–13) with \textit{COL7A1} mutations that created premature termination codons (PTCs) due to nonsense or splice-site mutations (Sp), small insertions or deletions. Another nine RDEB patients (patients 14–22) had missense mutations (Mis) in one allele of \textit{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 \textit{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 \textit{et al.}, 2004). As summarized in Table 2 and Supplementary on-line Figures 5S, there is 100% correlation between ELISA and immunoblotting results.

To determine if RDEB sera recognize C7 in the skin, we performed indirect immunofluorescence staining using salt-split human skin as substrate (Woodley \textit{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 \textit{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 \textit{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 in 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 et al., 2008, Wagner et 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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| 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           | P1523H6X187 / 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 / 6501G-A | TH / TH           | Mis / In-frame Del | RDEB-I            |
| 15         | 23          | R578X / G1907D | Fn3 / TH         | PTC / Mis     | RDEB-I            |
| 16         | 28          | IVS66+1G>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 (formerly 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 |
|------------|---------------------|-------------------------|---------------|-----------|-----------------|---------|
| 1          | Reduced             | +                       | +/-           | +         | +               | NC1/NC2 |
| 2          | Absent              | 0                       | -             | -         | -               | -       |
| 3          | Absent              | 0                       | -             | -         | -               | -       |
| 4          | Reduced             | 0                       | -             | -         | -               | -       |
| 5          | Reduced             | ++                      | +             | +         | +               | NC1/NC2 |
| 6          | Reduced             | +++                     | +             | +         | +               | NC1/NC2 |
| 7          | Reduced             | ++                      | -             | -         | -               | -       |
| 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 cryosections 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 assessed 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.