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Acute or Chronic Topical Retinoic Acid Treatment of Human Skin In Vivo Alters the Expression of Epidermal Transglutaminase, Loricrin, Involutrin, Filaggrin, and Keratins 6 and 13 but not Keratins 1, 10, and 14

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Histologic and immunocytochemical analyses were performed on cutaneous biopsies from 10 patients treated with retinoic acid under occlusion for 4 d compared to biopsies from 19 patients treated nightly for 16 weeks. Acute application of RA caused epidermal thickening (9 of 10 samples), stratum granulosum thickening (7 of 10), parakeratosis (4 of 10), a marked increase in the number of cell layers expressing epidermal transglutaminase (7 of 10), and focal expression of two non-epidermal keratins, K6 (8 of 10) and K13 (2 of 10), changes also observed with chronic treatment. Involutrin, filaggrin, and loricrin were also altered in samples from both acute and chronic treatment. An increased number of cell layers expressed both involucrin and filaggrin from both the acute (7 of 10) and chronic (14 of 19) treatment groups. In the acute group, loricrin expression was significantly reduced or absent in some regions of the epidermis (5 of 10), whereas most chronic samples showed an increased number of cell layers expressing loricrin (12 of 19). The pattern of expression of three major epidermal differentiation products, keratins K1, K10, and K14, was not significantly altered in any of the acute or chronic samples, although there was a slight reduction in the detection of K10 in two of the acute samples. Thus, acute topical RA treatment under occlusion caused substantial changes in the epidermis, and reproduced most, but not all of the effects of chronic treatment. J Invest Dermatol 98:343–350, 1992

Retinoids play an important role in the coordinated program of gene expression in keratinocytes. In cultured human epidermal cells, removal of vitamin A from the culture medium induces the synthesis of keratins K1 and K10, as well as transglutaminase-catalyzed crosslinking of the membrane proteins of the cornified envelope [1,2]. In cultured mouse and human keratinocytes, retinoids suppress a variety of morphologic and biochemical properties of differentiation [2–7]. In addition, retinoid excess in keratinocyte cultures induces the synthesis of two new keratins, K13 and K19 [8–11], normally expressed in internal stratified epithelia (K13) [12,13], mucus-secreting epithelia (K19) [13–15], or squamous cell carcinomas (K13 and K19) [16,17].

In vivo, topical applications of retinoids on rodents and humans cause epidermal thickening and hyperplasia [7,18,19]. Chronic topical application of all-trans retinoic acid (Retin-A, RA) also alters the expression of several markers of epidermal differentiation in photoaged human skin. These alterations include an increase in the number of cell layers expressing epidermal transglutaminase, and the induction of keratins K13 and K6, the latter of which is specific for hyperproliferation [20]. In contrast to the response of cultured keratinocytes to RA, topical RA did not appreciably suppress keratins K1 and K10 in the suprabasal layers, nor did it suppress K14 in the basal layer of the epidermis as detected by immunofluorescence [20].

To develop a model with a shorter time course, we determined whether the changes in marker expression induced by chronic RA treatment of photoaged skin could be reproduced by the application of RA under occlusion for 4 d. Skin biopsies derived from volunteers treated with 0.1% RA were examined histologically and immunocytochemically for the same markers of differentiation examined in the earlier study, which utilized patients treated for 16 weeks. In addition, three other markers of differentiation — involucrin, filaggrin, and loricrin — were examined in all the acute samples and 19 sets of samples from the original study. Our results indicate that for all markers except loricrin, the changes induced by acute RA treatment were consistent with those observed following chronic treatment. The detection of loricrin was reduced in the acute samples, but greatly enhanced in the chronic treatment group. Furthermore, many of the RA-induced alterations in both treatment groups differ from those in cultured keratinocytes [2–7], suggesting that although RA exerts some of its effects in vivo through its direct interaction with keratinocytes, alterations in the dermis and other

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Abbreviations:
RA: retinoic acid
VEH: vehicle control

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Table I. Histologic Changes with Chronic and Acute Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Individuals</th>
<th>Epidermal Thickening</th>
<th>Stratum Granulosum Thickening</th>
<th>Parakeratosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Rx</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>10</td>
<td>&lt; 0.1 mm</td>
<td>2/10</td>
<td>2/10</td>
</tr>
<tr>
<td>Strong</td>
<td>9</td>
<td>&gt; 0.1 mm</td>
<td>8/9</td>
<td>7/9</td>
</tr>
<tr>
<td>TOTAL</td>
<td>19</td>
<td></td>
<td>10/19</td>
<td>9/19</td>
</tr>
<tr>
<td>Acute Rx</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>4</td>
<td>&lt; 0.1 mm</td>
<td>1/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Strong</td>
<td>6</td>
<td>&gt; 0.1 mm</td>
<td>6/6</td>
<td>3/6</td>
</tr>
<tr>
<td>TOTAL</td>
<td>10</td>
<td></td>
<td>7/10</td>
<td>4/10</td>
</tr>
</tbody>
</table>

* From [20].

Based on epidermal thickness.

RESULTS

Histologic Analysis Nine of ten of the RA-treated samples showed an increase in epidermal thickness above controls. As with chronic treatment [20], the ten individuals in the present study fell into two groups. Thickening was particularly pronounced in six of the RA-treated samples (> 0.1 mm), designated “strong responders,” whereas the four “weak responders” showed less epidermal thickening above controls. Epidermal thickening was due to an increase in the total number of cell layers, as well as an increase in the size of some of the individual cells. Seven of the ten RA-treated samples showed a marked increase in the number of granular cell layers above controls, whereas four of the ten treated skins demonstrated parakeratosis (Fig 1; Table I). As with the chronic treatment group, there was a significant association between epidermal thickening and granular layer thickening (p = 0.03), but in contrast to the previous study [20] parakeratosis was not associated with the strong responder group (p = 0.57; two-tailed Fisher’s exact test), and was not as severe.

Immunocytochemical Analysis Antibodies to K1 detected this keratin in the living suprabasal layers of the epidermis of control samples, whereas K14 was detected in all living cell layers, although most strongly in the lower layers. In double-labeling experiments, only K14 is detected in the basal layer, whereas K1 and K14 are co-expressed in all spinous and granular layers (Fig 2). RA-treated skin showed this same pattern of expression for these two keratins (Fig 2). As with the chronic treatment group [20], the skin treated for 96 h did contain more cell layers co-expressing K1 and K14, consistent with an increase in the number of suprabasal cells. K1 and K10 are co-expressed in all suprabasal layers of all normal and treated epidermis in the acute-treatment group (Fig 3), although there was a slight reduction in the stain intensity of K10 in two of the RA-treated samples.

Epidermal transglutaminase is localized primarily in cell membranes of the stratum granulosum and stratum corneum in control

Figure 1. Analysis of control and RA-treated skin biopsies. Hematoxylin-eosin staining of 4-d vehicle-treated (top), and RA-treated (bottom) skin sections from one patient from strong responder group. Bar, 0.1 mm, for both top and bottom panels (magnification × 100).

components of the skin may play an important role in the epidermal response to RA [21,22].

MATERIALS AND METHODS

The protocol was approved by the University of Michigan Institutional Review Board and patients provided informed consent. Four-millimeter punch biopsies were taken from the hips or buttocks of 10 normal volunteers following topical application of RA or vehicle alone, thus providing one control and one experimental sample per patient. Vehicle (0.5 g/9 cm² area) with or without 0.1% RA cream (Retin-A, Ortho Pharmaceutical Corp., Raritan, NJ) was applied topically under a plastic wrap (Saran Wrap) occlusion, fastened with surgical tape, for 96 h. The biopsies were placed in O.C.T. compound (Miles Scientific), and quick frozen in liquid nitrogen that was allowed to evaporate at −70°C. Punches were removed from −70°C, placed in dry ice, and sent to the National Cancer Institute. For the chronic treatment samples, punch biopsies were derived from forearm skin treated nightly with 0.1% RA cream or vehicle control for 16 weeks, as described [20].

Frozen 5-μm biopsy sections were either stained with hematoxylin and eosin, or incubated overnight with rabbit antiserum (involucrin), affinity-purified rabbit anti-peptide antisera (keratins K6, K10, K14, and loricin), guinea pig anti-peptide antisera (K1 and K14), mouse monoclonal culture supernatant (K13 and epidermal transglutaminase), or mouse monoclonal antibody from ascites fluid (filaggrin) as described [20]. For involucrin and filaggrin staining, slides were first fixed in 10% formalin for 15 min. Antisera against K1, K6, K10, and loricin were generated against C-terminal peptides of published sequences [23–27]; K14 anti-peptide antisera was generated similarly as described [28]. Involution and filaggrin antibodies were obtained from Biomedical Technologies, Inc. (Stoughton, MA). Involution antisera was used at a dilution of 1:50, filaggrin at 1:16,000, and loricin at 1:500. All other procedures for single and double indirect immunofluorescence were performed as described previously [20].

Epidermal thickness was assessed from hematoxylin-plus-eosin-stained sections using a calibrated optical micrometer, as described [20].

Statistical Methods Fisher’s two-tailed exact test [29] was used for all comparisons of proportions.
**Figure 2.** Double indirect immunofluorescence of vehicle control (top row) and RA-treated (bottom row) skin sections from one individual. Magnification ×50. Enlarged to the same extent as Figs 3 and 4 for size comparison. K14 and K1. Sections were stained using K14-specific rabbit antisera (green fluorescein staining, left column), and K1-specific guinea pig antisera (Texas Red staining, middle column). Right column: double exposure of K14/K1 staining. Note single layer positive for K14 (green) and suprabasal layers positive for both K14 and K1 (yellow). The epitope recognized by the K1 antisera is not present in cornified layers. Control experiments reveal that staining in the stratum corneum is the result of non-specific trapping of reagent.

**Figure 3.** Double indirect immunofluorescence of vehicle control (top row) and RA-treated (bottom row) skin sections from individual of Fig. 2. Magnification as in Fig 2. K10 and K1. Sections were stained using K10-specific rabbit antisera (green fluorescein staining, left column), and K1-specific guinea pig antisera (Texas Red staining, middle column). Right column: double exposure of K10/K1 staining, showing coexpression of K1 and K10 in all suprabasal layers.
Table II. Immunochemical Changes with Chronic and Acute Treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>K6</th>
<th>K13</th>
<th>Expanded &amp;tase</th>
<th>Expanded Involutrin</th>
<th>Expanded Filaggrin</th>
<th>Expanded Loricrin</th>
<th>Discontinuous Loricrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic Rx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Weak</td>
<td>2/10</td>
<td>1/10</td>
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<td>5/10</td>
<td>9/10</td>
<td>5/10</td>
<td>0/10</td>
</tr>
<tr>
<td>Strong</td>
<td>7/9</td>
<td>4/9</td>
<td>8/9</td>
<td>9/9</td>
<td>9/9</td>
<td>9/9</td>
<td>0/9</td>
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<tr>
<td>TOTAL</td>
<td>9/19</td>
<td>5/19</td>
<td>10/19</td>
<td>14/19</td>
<td>14/19</td>
<td>14/19</td>
<td>0/19</td>
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<tr>
<td>Acute Rx</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak</td>
<td>2/4</td>
<td>1/4</td>
<td>1/4</td>
<td>1/4</td>
<td>0/4</td>
<td>0/4</td>
<td>1/4</td>
</tr>
<tr>
<td>Strong</td>
<td>6/6</td>
<td>1/6</td>
<td>6/6</td>
<td>6/6</td>
<td>3/6</td>
<td>3/6</td>
<td>4/6</td>
</tr>
<tr>
<td>TOTAL</td>
<td>8/10</td>
<td>2/10</td>
<td>7/10</td>
<td>7/10</td>
<td>3/10</td>
<td>3/10</td>
<td>5/10</td>
</tr>
</tbody>
</table>

* From [20].
† Based on epidermal thickness as in Table I.
‡ Samples negative for K6 are also negative for changes in other markers.
§ The same sample shows expanded &tase, involucrin, and filaggrin, discontinuous loricrin, and stratum granulosum thickening.
\* One sample displays simultaneous vertical expansion and discontinuous expression.

skin sections (Fig 5) [30]. There was a marked expansion (more than one fourth of the total thickness of the living layers) of epidermal transglutaminase-positive cells towards the lower layers of the epidermis in seven of ten samples treated acutely (Fig 5; Table II). As with chronic treatment, the pattern of epidermal transglutaminase expression was significantly associated with epidermal thickness (p = 0.033). The cell-membrane—specific localization of epidermal transglutaminase was consistent between all control and experimental acute and chronic treatment groups.

Involucrin, filaggrin, and loricrin expression were examined in the ten sets of acute samples, as well as 19 samples from the chronic treatment group [20]. These markers had not been examined in the previous study of chronic treatment. In control samples, these three markers are all expressed in the upper layers of the epidermis. Involucrin, which was present in the uppermost spinous layers, as well as the granular and cornified layers, showed a pattern of expression similar to epidermal transglutaminase. Filaggrin was localized to the stratum granulosum and stratum corneum, whereas loricrin was more tightly restricted to the one to three cell layers of the stratum granulosum in the control group (Figs 4 and 5).

In acute and chronic RA-treated epidermis both involucrin and filaggrin were expressed in substantially lower layers of the epidermis (more than one fourth the thickness of the total living layers); double immunofluorescence demonstrated that these two markers were always coordinately expanded (p < 0.00001). Although, as in control skin, involucrin was detected in lower layers than filaggrin (Fig 4). In five RA-treated biopsies, involucrin expression extended to the basal layer of the epidermis, whereas filaggrin could be detected in the mid to lower region of the spinous layer. Seven of ten of the acute, and 14 of 19 of the chronic treatment group showed an expansion of both of these markers (Table II); this pattern was significantly associated with the strong responder group in both the acute (p = 0.033) and chronic treatment groups (p = 0.033), as well as with the enhanced expression of epidermal transglutaminase (p = 0.0018 for chronic; p = 0.0006 for acute).

In the chronic treatment group, loricrin expression was markedly expanded towards the basal layer (two- to fivefold increase above control) in 12 of 19 samples (Fig 5; Table II). This pattern of expression was significantly associated with both epidermal thickness (p = 0.003) and the expansion of layers positive for involucrin and filaggrin (p = 0.0018). In the acute treatment group, however, regions of loricrin-positive granular cells were interrupted by loricrin-negative areas in five of ten samples (Fig 5; Table II), suggesting either a reduction in loricrin expression or stability, or a masking of the epitope. These loricrin-negative regions ranged from one eighth to one half of the entire length of the section. In contrast, loricrin-positive granular cells formed a more contiguous stretch in control epidermis. Only three of ten samples from the acute-treatment group displayed a vertical expansion of loricrin expression similar to that seen in the chronic-treatment group, although one of the three also showed a patchy distribution. Neither horizontal inhibition (p = 0.52) nor vertical expansion (p = 0.20) of loricrin were correlated with epidermal thickness.

In control sections of both acute- and chronic-treatment groups, keratin K6 immunostaining was almost exclusively limited to the hair follicle outer root sheath (Fig 6) [15,31], although in two 4-d control samples the interfollicular epidermis stained faintly for this keratin. In contrast, K6 was observed in interfollicular keratinocytes of eight of the ten 4-d RA-treated samples, as compared to only nine of 19 in chronic treatment (Fig 6; Table II). The pattern of expression for this keratin was patchy, and usually limited to the suprabasal layers in both groups, although more K6-positive cells were observed in the skin samples from the acute-treatment group (Fig 6). All six strong responders expressed K6, whereas two of the weak responders expressed this keratin. Therefore, in contrast to the chronic-treatment group, K6 expression was not significantly associated with epidermal thickness (p = 0.133; Table II).

K13-specific antibodies also did not detect the presence of this keratin in any of the control samples. However, in two of the ten acute RA-treated skins, focal expression of K13 was seen in the suprabasal layers (Fig 7; Table II). As with 16-week RA treatment, K13 expression also did not correlate significantly with the magnitude of the histologic response or the expression of the other markers.

DISCUSSION

The alterations in several markers induced by chronic topical RA described in the previous study [20], as well as in the present study, can be reproduced by a single application of RA under an occlusive dressing. These changes include epidermal thickening (19 of 19 for chronic versus nine of ten for acute), granular layer thickening (10 of 19 versus seven of ten), parakeratosis (nine of 19 versus four of ten), induction of K6 (nine of 19 versus eight of ten), and K13 (five of 19 versus two of ten), and expanded expression of epidermal transglutaminase (12 of 19 versus seven of ten), involucrin, filaggrin (both 14 of 19 versus seven of ten), and loricrin (12 of 19 versus three of ten). However, keratins K1, K10, and K14 retained the normal pattern of expression in all samples from both the acute- and chronic-treatment groups.

In both the acute and chronic groups there was a significant association between the magnitude of the histologic response, as defined by epidermal thickness, and the altered expression of epidermal transglutaminase, involucrin, and filaggrin. The only major difference between the two treatment groups resides in the pattern of expression of loricrin. The precocious expression of loricrin was correlated with the other markers only in the chronic group, whereas in the acute group detection of this marker was reduced in five of ten cases, even when the other markers were enhanced, although this may be due to masking of loricrin because of increased cross-linking. Another difference between treatment groups was the detection of keratin K6 in a greater percentage of the acute
Figure 4. Double indirect immunofluorescence of vehicle control (top row) and RA-treated (bottom row) skin sections from individual of Fig 2. Magnification as in Fig 2. Involucrin and filaggrin. Sections were stained using involucrin-specific rabbit antiserum (green fluorescein staining, left column), and mouse monoclonal antibody (BC-1) specific for filaggrin (Texas Red staining, middle column). Right column: double exposure of involucrin/filaggrin staining. Note expression of both markers in spinous layers in RA-treated samples (bottom row).

Figure 5. Double indirect immunofluorescence of 4-d vehicle control (top) and 4-d RA-treated skin sections (middle) from one individual, and 4-month RA-treated skin from another individual (bottom). Loricrin and epidermal transglutaminase sections were stained using loricrin-specific rabbit antiserum (green fluorescein staining, left column), and mouse monoclonal antibody specific for filaggrin (Texas Red staining, middle column). Right column: double exposure of loricrin/epidermal transglutaminase. Note that loricrin is suppressed in the 4-d sample and enhanced in the 4-month sample, whereas transglutaminase is expanded in both groups. The 4-month vehicle control sample showed the same pattern of staining as that for the 4-d sample (not shown).
Figure 6. Double indirect immunofluorescence of K14 and K6. Sections were stained using K14-specific guinea pig antisera (Texas Red) and K6-specific rabbit antisera (fluorescein). Double exposure of K14/K6 staining in 4-d (top) and 4-month (bottom) treatment groups. Left: vehicle control, showing K6 (yellow) localized to the hair follicle (×25). Center: RA-treated epidermis, showing K6 expression in interfollicular epidermis (×25). Right: RA-treated epidermis (×50). Note increased number K6-positive suprabasal cells in 4-d treatment group.

Figure 7. Double indirect immunofluorescence of K14 and K13 in 4-d RA-treated skin sections from two individuals. Top row: Individual 1. Bottom row: Individual 2. Sections were stained using K14-specific rabbit antisera (green fluorescein staining, left column), and mouse monoclonal antibodies (IC7 and 2D7) specific for K13 (Texas Red staining middle column). Right column: double exposure of K14/K13 staining, showing suprabasal and focal nature of K13 expression in both individuals. No K13 staining was observed in any control sections.
versus the chronic group. The reason for these differences is unclear, although a higher dose resulting from the occlusion treatment in the acute-treatment group, along with a potential tachyphylactic response to RA during chronic treatment may contribute to observed differences. However, these explanations imply a differential response of certain markers to RA.

Immunofluorescent analysis of these markers of differentiation has the advantage of being a highly specific and sensitive technique for the detection of changes in gene expression at the protein level. The detection of new markers, such as K6 and K13, or the detection of markers in additional cell layers, such as epidermal transglutaminase, involucrin, and filaggrin, is likely to represent bona fide changes in gene expression. On the other hand, the reduced detection of a marker, as in the case of loricin, may represent the loss of antigenicity rather than suppression of the marker itself. In addition, small changes in gene expression are difficult to assess by this technique, as may be the case for keratins K1, K10, and K14. However, our preliminary in situ hybridization experiments indicate that there are no major changes in K1 mRNA levels for at least two of the individuals, representing one strong and one weak responder from the acute treatment group, following exposure to RA (data not shown).

The previously reported effects of chronic RA [20], and acute RA as reported in the present study on certain markers of epidermal differentiation in vivo, are for the most part different than those seen with RA treatment of cultured human keratinoctyes. The precocious induction of epidermal transglutaminase is in contrast to the suppressive effects of RA on epidermal transglutaminase activity and cornified envelope formation in cultured keratinocytes [2], although we have measured antigenicity as opposed to enzyme activity. In addition, the suppression of cornified envelope formation in culture may be at least in part due to the in vitro suppression of loricin [32], which is the major component of the cornified envelope [27]. The absence of any gross changes in the patterns or levels of K1, K10, and K14 expression is in contrast with the suppression of these markers in vitro by RA. The alterations in the additional markers used in the present study also illustrate the contrast between the two systems. Involucrin and filaggrin were induced in both the acute- and chronic-treatment groups; in cultured keratinocytes, however, filaggrin is inhibited whereas involucrin is for the most part unaffected by RA, whether cells are cultured on plastic or dermal substrates [23,33,34]. On the other hand, the induction of K13 in both the acute- and chronic-treatment groups, and the suppression of loricin in the acute-treatment group, were consistent with the response of cultured keratinocytes to RA [8–11, 32].

The differential effects of RA on marker expression in vivo and in culture may ultimately stem from the interaction of keratinocytes with a vast network of other epidermal and dermal components. This could result in the elaboration of extracellular matrix components, such as fibronectin, that control differentiation [35], or different families of growth stimulatory and inhibitory factors derived from both the dermis and epidermis [36,37] that have been shown to differ in their abilities to suppress markers of differentiation [38]. It is also possible that gradients of factors that modulate differentiation, such as calcium [39,40], endogenous retinoids [41], and the families of cytosolic retinoid binding proteins [42,43] and nuclear receptors [44] that mediate their effects may be altered by topical RA treatment. In light of the major differences between the effects of RA on epidermal differentiation in vitro and in vivo, caution should be exercised when attempting to predict the in vivo response to RA by extrapolation of results from cell-culture experiments.

Some of the observed changes in marker expression, such as the induction of K6, increased epidermal and stratum granulosum thickness, and parakeratosis are consistent with retinoid-induced hyperplasia in vivo [45–51]. However, the observed patterns of expression for certain markers are clearly not the result of hyperplasia alone. This includes the suppression of loricin in the acute-treatment group, and the induction of K13 in both treatment groups, as these changes do not occur in various forms of acute and chronic epidermal hyperproliferation in vivo [52,53]. In cultured epidermal cells, retinoids also suppress loricin [32] and induce K13, but not the hyperproliferative keratin K6 [8,9,11], suggesting that loricin suppression and K13 induction are unrelated to the hyperproliferative response in vivo.

These results indicate that both acute and chronic RA causes substantial changes in gene expression; some of these retinoid-related changes may be a consequence of hyperplasia whereas others may result from more specific effects of RA on both the dermis and epidermis. Further studies will elucidate the changes in physiology that accompany these immunohistochemical changes.

We are grateful to Scott Thacher (Texas A & M) for the kind gift of mouse monoclonal anti-epidermal transglutaminase antibody (BC-1), and also to Goos van Muijen (Katholieke Universiteit; Nijmegen, Netherlands) for the kind gift of mouse monoclonal anti-K13 antibodies (IC7 and 2D7). We are indebted to Margaret Taylor for the typing of this manuscript.

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