Differences in Involucrin Immunolabeling Within Cornified Cell Envelopes in Normal and Psoriatic Epidermis

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Epidermal keratinocytes form a cornified cell envelope (CE) beneath the plasma membrane during the late stages of differentiation. This CE is stabilized by cross linking of several precursor proteins, including involucrin. In psoriasis, the expression pattern of the precursor proteins is known to be deranged; involucrin expression is increased and loricrin expression is decreased. However, these changes have not been previously evaluated ultrastructurally. In the present study, we performed light and electron microscopic immunohistochemistry in conjunction with conventional transmission electron microscopy to assess the nature of involucrin involvement in normal and psoriatic CE. In normal epidermis, CEs were observed from the deepest cornified cells or, when present, from the transitional cells, increasing in thickness and changing electron densities with maturation. In psoriatic epidermis, CE formation started earlier, one to several cells below the cornified layer. Psoriatic CEs were generally thinner and showed a constant high electron density. Immunoelectron microscopy revealed that the normal CE was involucrin positive only at a very early stage, whereas psoriatic CE showed persistent involucrin immunoreactivity. These results suggest that in normal skin, involucrin is the major constituent of the CE only in its early stages of assembly. In contrast, CE formation seems to be initiated prematurely in psoriatic skin, where involucrin remains the major constituent of the CE during maturation. *Key words: keratinocytes/immunoelectron microscopy/keratinization/skin. J Invest Dermatol 104: 391–395, 1995*

The cornified cell envelope (CE) is a highly insoluble structure that is formed beneath the plasma membrane during the terminal differentiation of keratinocytes [1–3]. Normally, it is 10–16 nm in thickness [4,5] and is stabilized by the cross linking of various proteins, including involucrin, loricrin, and small proline-rich proteins (or cornifin or pancornulin) [1–3]. Calcium-dependent transglutaminase(s) is responsible for the formation of γ-glutamyl-ϵ-lysine isopeptide bonds between these molecules (for a review, see [1]). It has been suggested that the CE forms as a result of sequential assembly processes involving the specific precursor proteins at different times [6].

Psoriatic epidermis is characterized by increased keratinocyte proliferation and defective keratinization, which includes altered formation of the CE. Previous ultrastructural assessments of plasma membranes and CEs in psoriasis have reported thin, incomplete, or poorly prominent membranous structures [7–9], but have given little specific information about keratinocyte terminal differentiation in psoriasis.

Apart from the different morphology [10], psoriatic CE shows a markedly different biochemical composition compared to normal CE [11]. The former is relatively close to that of involucrin, whereas the latter is closer to that of loricrin. Although both involucrin and membrane-bound transglutaminase, which is responsible for the initial CE assembly, are expressed precociously in psoriasis [12–15], it remains to be determined whether the formation of the CE itself is actually premature, because the assembly process of CE in psoriatic epidermis has not been evaluated with respect to the ultrastructural localization of the involucrin. To address this question, we performed light and electron microscopic immunohistochemistry and conventional transmission electron microscopy on involucrin in normal and psoriatic skin.

MATERIALS AND METHODS

Skin samples were taken from the center of typical chronic psoriatic plaque lesions on the forearm, shin, knee, or lumbar area from five patients (2 men and 3 women, ages 57 to 77 years) under local anesthesia after informed consent. No topical treatment had been applied for at least 1 week before the skin biopsy, and no patients were receiving oral retinoids or other systemic therapy. Control samples, consisting of normal human skin from the neck, chest, and thigh, were obtained during surgical operations for benign skin tumors.

For transmission electron microscopy, small pieces of skin were fixed in 5% glutaraldehyde in 0.08 M cacodylate buffer, pH 7.4, or in half-strength Karnovsky fixative [16], followed by further fixation in 1% osmium tetroxide in distilled water. After *en bloc* staining with uranyl acetate, the specimens were dehydrated in ethanol and embedded in Araldite (Oken, Tokyo, Japan) or Epon 812 (Taab, Berkshire, UK). Ultrathin sections were stained with uranyl acetate and lead citrate.

For light microscopic observation, 6-μm cryostat sections of snap-frozen samples were air-dried and fixed in acetone at 4°C for 10 min. After preincubation with 1% bovine serum albumin, 5% normal goat serum, and 0.1% gelatin in phosphate-buffered saline, pH 7.4, for 15 min at room temperature, the sections were incubated with one of two rabbit polyclonal

anti-involucrin antibodies—BT601 (Biomedial Technologies, Stoughton, MA) or DH1 (a generous gift from Dr. F.M. Watt, ICRF, London)—for 1 h at 37°C. After washing, the sections were incubated further with 1 nm colloidal-gold–conjugated secondary antisera (Amersham International, Amersham, UK) for 1 h at 37°C. Subsequently, the immunogold particles were enhanced using a silver staining kit (Amersham International). Sections were then stained briefly with methyl green for orientation.
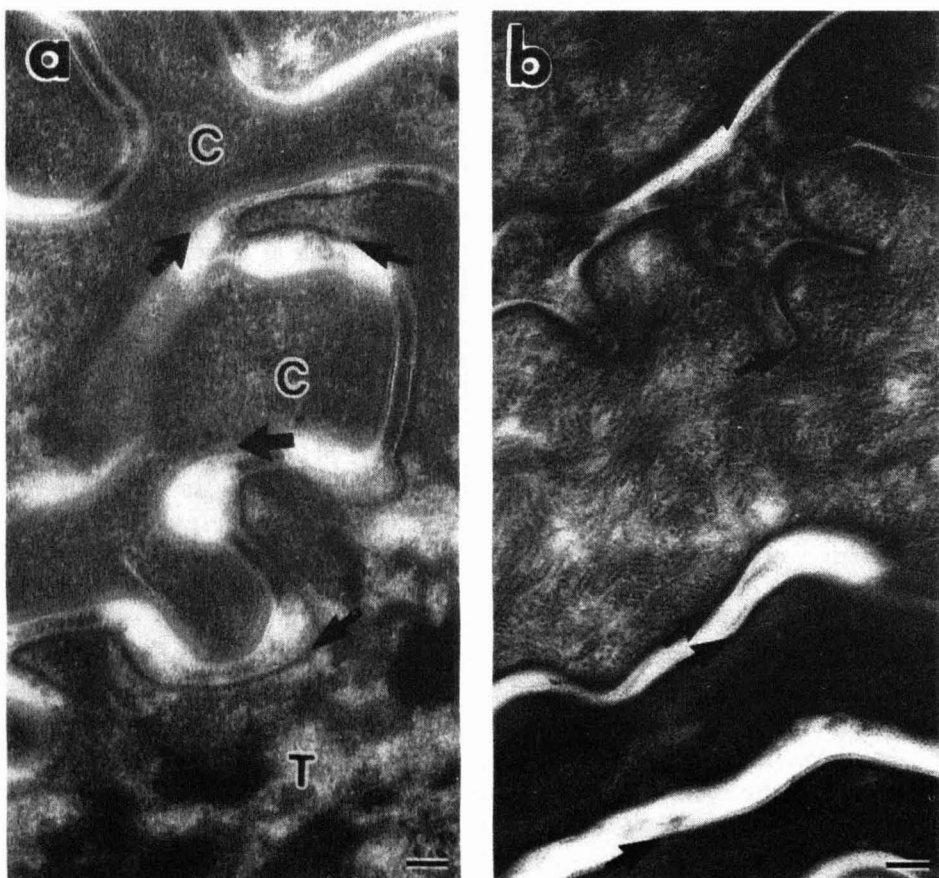
For post-embedding immunoelectron microscopy, tissues were fixed in 1% glutaraldehyde and 0.2% picric acid in phosphate-buffered saline, pH 7.4, for 3 h, dehydrated with ethanol, and embedded in LR White resin (The London Resin, Hampshire, UK). Ultrathin sections were cut and pre-incubated in the blocking buffer as described above, followed by incubation with one of the primary anti-involucrin antibodies. After washing, the sections were incubated with 10 nm colloidal-gold–conjugated secondary sera (Amersham International). The sections were then stained with uranyl acetate for contrast. For control studies, normal rabbit serum was used instead of the primary antibodies.
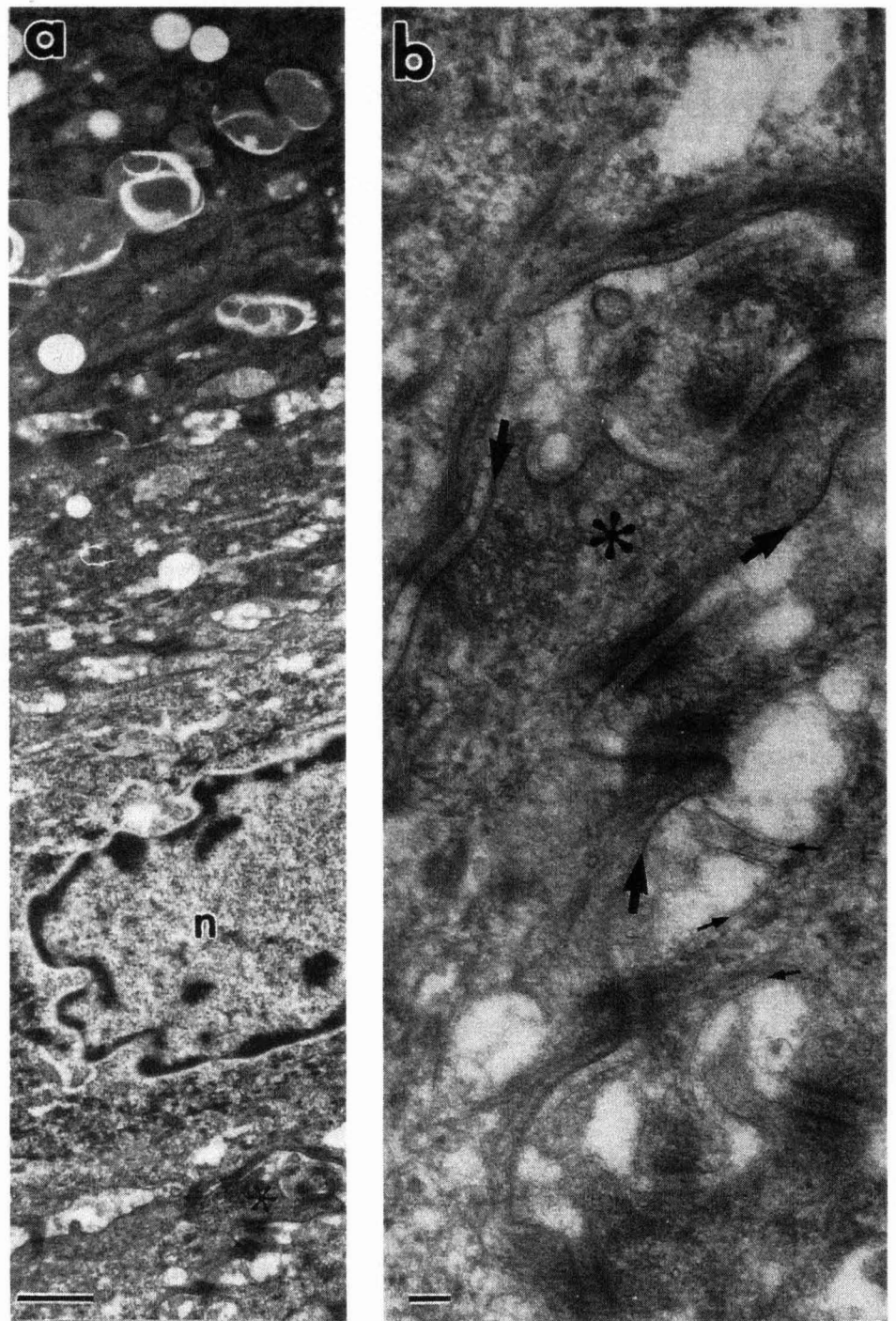
RESULTS

Electron Microscopy Ultrastructural examination of normal CE demonstrated gradual morphologic changes. CEs were usually observed from the deepest cornified cells. In these cells, the CEs were about 15–20 nm in thickness **(Fig 1a)**. Rarely, some transitional cells were present between the granular and the cornified cells. In these transitional cells, the morphologic features of the CEs were different, appearing thinner (about 10 nm in thickness) and of higher electron density than the CEs of the overlying cornified cells. The electron density of the normal CEs within cornified cells became higher again in the middle horny layer **(Fig 1b)**.

In contrast, in the psoriatic epidermis, CE formation was observed from the upper or middle epidermal, living (spinous) cell layers **(Figs 2 and 3)**. These keratinocytes possessed morphologically normal-looking nuclei and intact cell organelles such as mitochondria and rough endoplasmic reticulum. The CE in these cells was about 10 nm in thickness and electron dense. The same observations were made in the cornified cell layers. The changes were most apparent in psoriatic lesions showing parakeratosis with absent granular layers.

Immunohistochemistry Immunohistochemistry revealed distinct distribution patterns of involucrin in normal and psoriatic
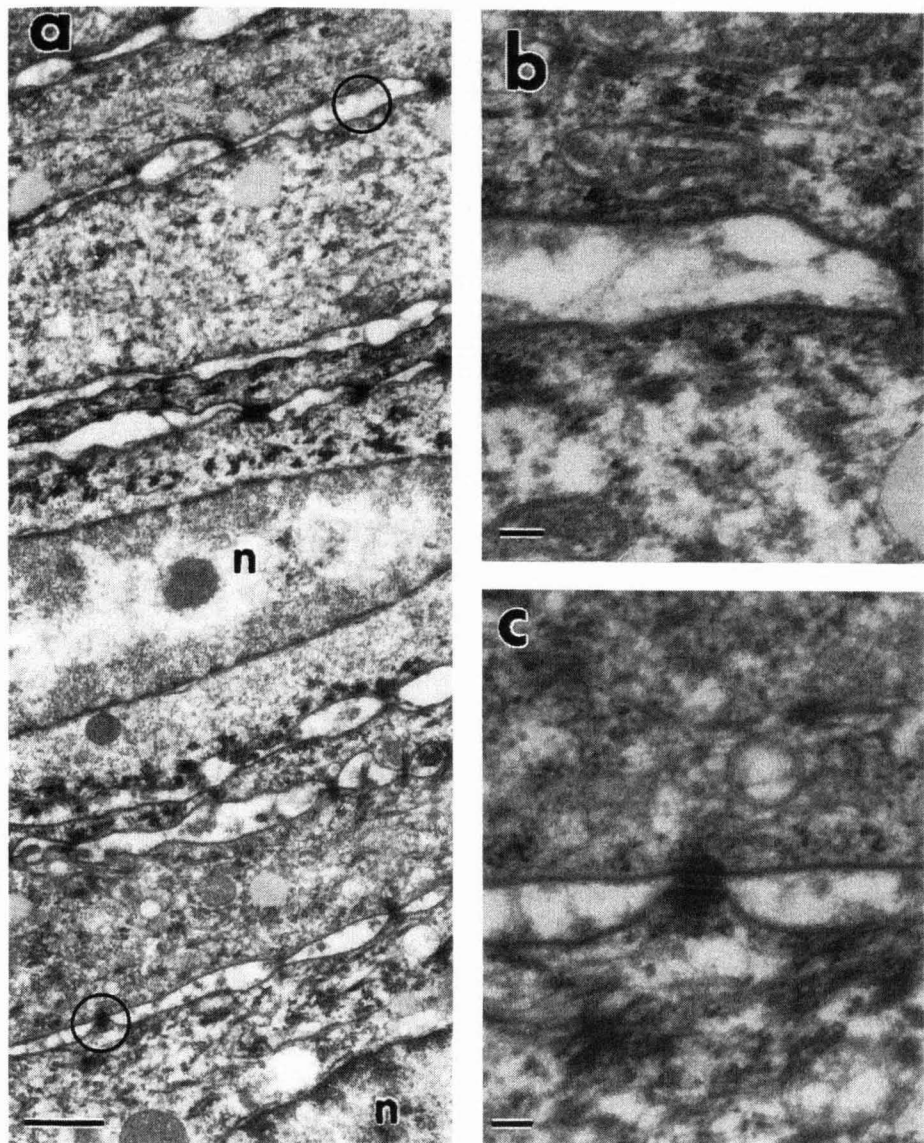

Figure 2. CEs in psoriatic skin. *a*) Relatively gradual cytologic changes from a nucleated living keratinocyte (*bottom*) to a cornified cell (*top*). The area depicted by an *asterisk* is shown at higher magnification in (*b*). *b*) CEs (*large arrows*) are thicker than the plasma membrane without CEs (*small arrows*), although the former is still thinner than the CEs in cornified cells of normal skin (compare with **Fig 1**). n, nucleus. *Bars, a,* 1 μm; *b,* 0.1 μm.

epidermis. In normal skin, involucrin staining was mostly confined to the granular cell layer **(Fig 4a)**. In psoriatic skin, involucrin labeling was greater, extending from the midspinous layer of the epidermis to the cornified cell layer **(Fig 4b,c)**. In the psoriatic cornified cells, involucrin staining was prominent within the peripheral part of the cells. This observation was not made in normal skin.

Immunoelectron Microscopy Immunoelectron microscopy also demonstrated differences in involucrin distribution in normal and psoriatic epidermis. In normal epidermis, involucrin staining was seen diffusely within the cytoplasm of granular cells and lower cornified cells **(Fig 5a)**. CE-associated involucrin labeling was observed only occasionally in the deepest cornified cells or in the transitional cells. In psoriatic epidermis, diffuse intracellular and CE-associated involucrin labeling was seen in the living cell layers **(Fig 5b)**. In the cornified cells, involucrin staining was confined to the CEs, and intracellular staining was rare **(Fig 5c,d)**.

DISCUSSION

Although involucrin has been reasoned to be the main constituent of the CE in cultured keratinocytes [17], its function in normal *in vivo* epidermal keratinization is less clear. The amino acid composition of the normal CE is markedly different from that of involucrin, but is close to that of loricrin [3]. Indeed, the present and

Figure 1. CEs in normal skin. *a*) Lower and *b*) mid-stratum corneum. Note that the CE of a transitional cell (*a, small arrows*) is thinner and more electron dense than the corresponding CE of overlying cornified cells (*a, large arrows*). In the mid-stratum corneum, the CEs become electron dense again (*b, arrows*). C, cornified cells; T, transitional cell. *Bar,* 0.1 μm.
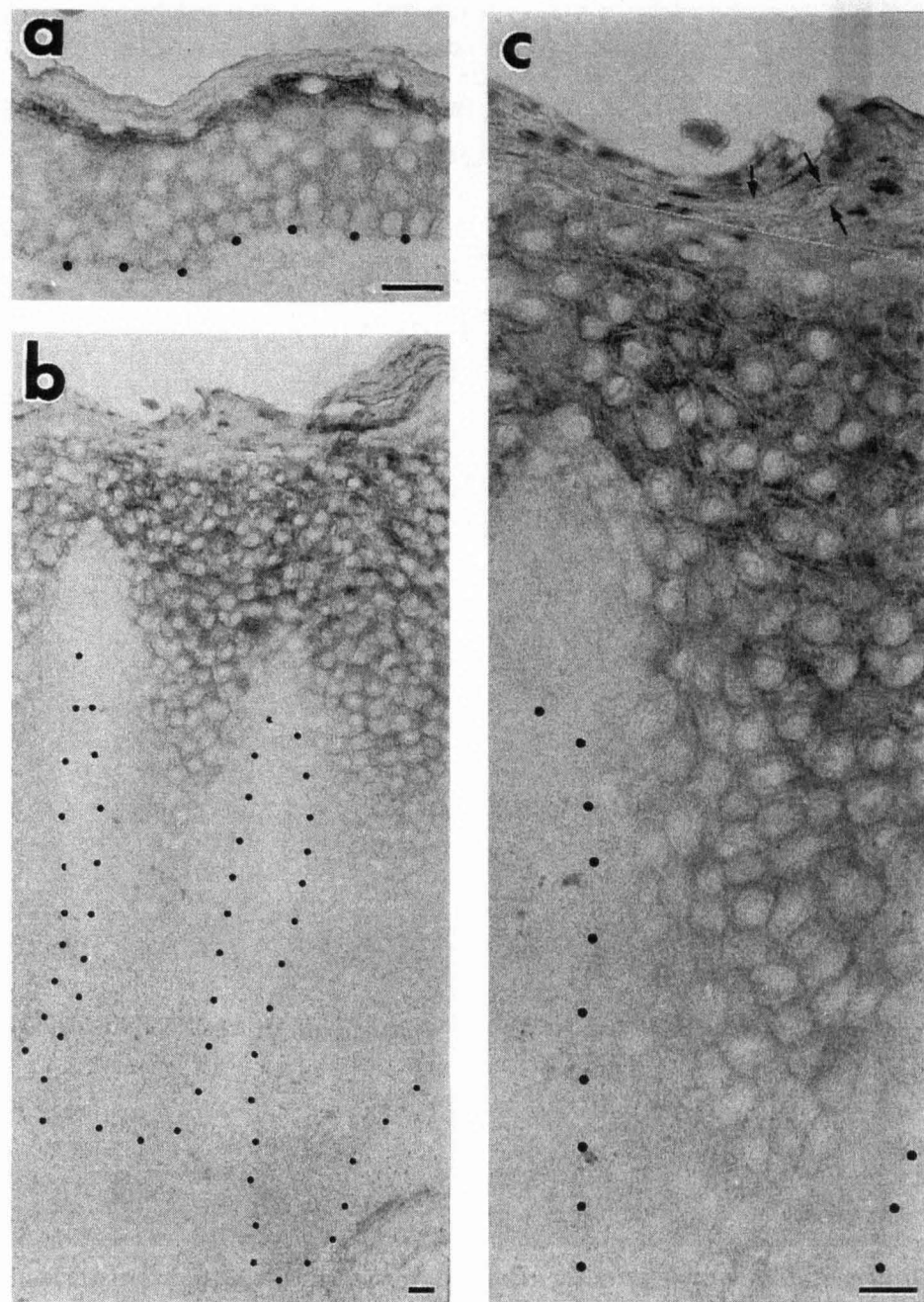
Figure 3. Psoriatic CEs demonstrating a lack of maturation. Two areas marked with *circles* (*a, top and bottom*) are enlarged in *b* and *c*, respectively. Psoriatic CEs remain electron dense and thinner than normal CEs (see **Fig 1**). n, nucleus. *Bars: a*, 1 μm; *b,c*, 0.1 μm.
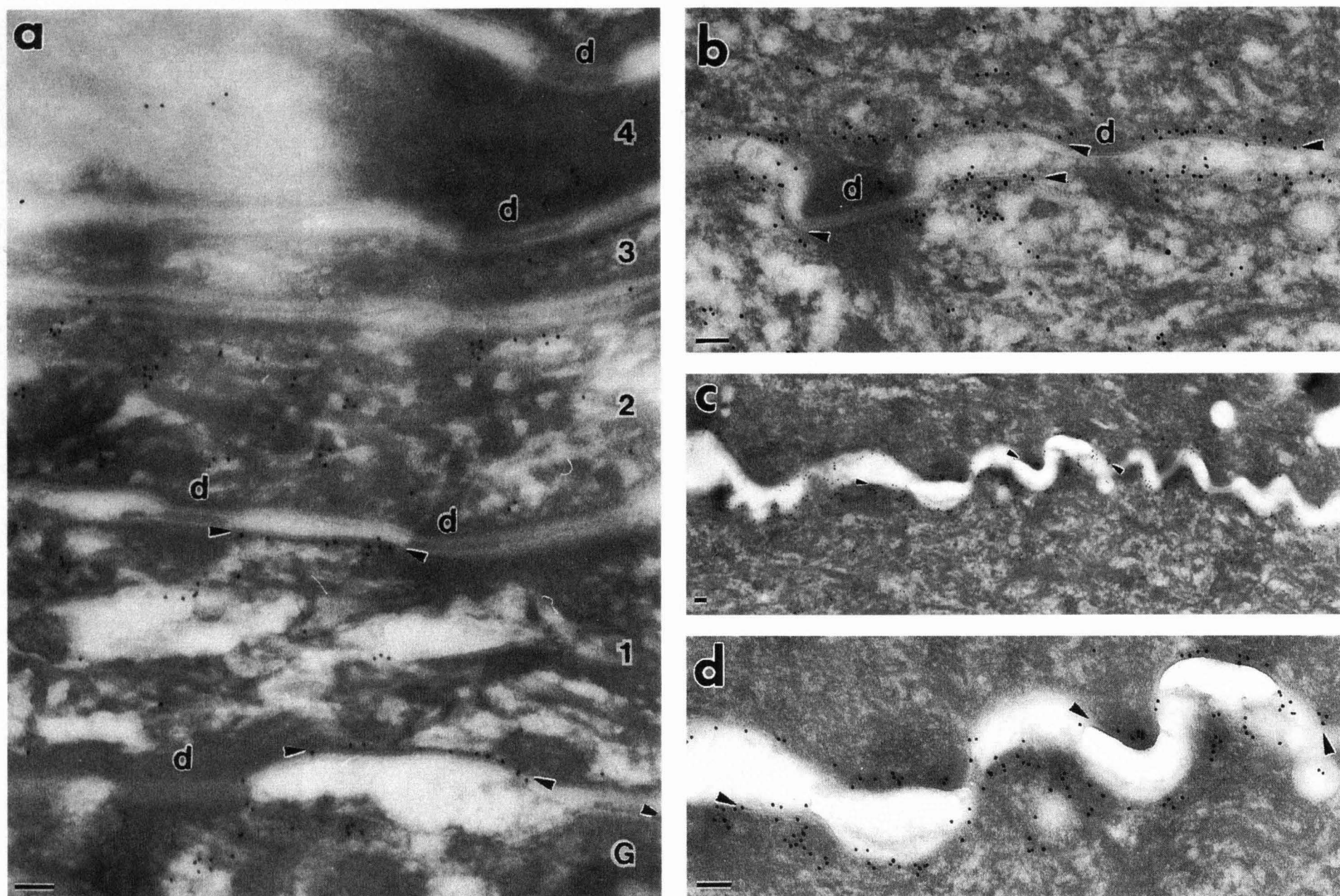

Figure 4. Involucrin (DH1 antibody) staining in normal and psoriatic epidermis. *a*) Normal skin (from the chest) shows involucrin staining in the stratum granulosum. *b*) In psoriatic epidermis, there is positive labeling extending from the mid-epidermis to the stratum corneum, shown at higher magnification in *c*. *c*) Note the peripheral staining (*arrows*) in the psoriatic cornified cells. *Dotted lines*, dermal-epidermal junctions. *Bars*, 20 μm.

previous immunoelectron microscopic studies [18–20] did not show intense involucrin labeling in the normal CE, whereas significant involucrin labeling was detected in the cytoplasm (**Fig 5a** and see [18,19]).

In the present study, the normal CE changed its involucrin immunoreactivity with maturation. CEs were involucrin immunoreactive only in the transitional cells or, rarely, in the deepest cornified cells. The CE of the more superficial cells lacked involucrin staining. This can be explained by masking of involucrin antibodies by other cross-linked CE-associated proteins, such as loricrin. In fact, heavy loricrin labeling on the normal CE has been reported [21]. Loricrin, the major constituent of the normal CE, is known to be expressed later than involucrin in keratinocyte maturation [22,23].

In our study, the ultrastructural changes of the CE correlated well with the involucrin staining pattern. The immature, thin CE with high electron density observed in the transitional cells by conventional electron microscopy corresponded to the involucrin-positive CE observed by immunoelectron microscopy. Because such transitional cells are observed infrequently in the normal human epidermis, maturation from the involucrin-positive CE to the involucrin-negative CE is rarely appreciated. Nevertheless, the presence of such sequential morphologic and immunocytochemical changes, as seen in these occasional transitional cells, suggests an ordered assembly process of the normal CE, which predominantly involves involucrin in its very early stage.

The present study also demonstrates unequivocally the precocious CE formation in psoriasis, in which the ultrastructurally appreciable CE was observed from the living cell layers. Similar CE formation in living cell layers was reported previously in mucous membranes such as the tongue, oral mucosa, and lip [5,24–27]. Whereas the normal CE became thicker and changed electron density during maturation (**Fig 1**) [4,28], the psoriatic CE remained

thin and of constant electron density. Furthermore, the involucrin immunoreactivity remained positive in psoriatic CE. This may be related to a decreased expression of loricrin in psoriasis [29], because the CE of other epithelia that lack loricrin, such as lip mucosa, also remains involucrin positive during its maturation (our unpublished observations).

In psoriatic cornified cells, virtually all the involucrin labeling was associated with CEs, whereas normal cornified cells showed substantial intracellular labeling. Thus, involucrin seems not to be incorporated fully into the CE of the normal skin. It remains to be determined whether the non–CE-associated involucrin molecules have some other function within the stratum corneum.

Eckert *et al* [6] proposed a two-step model for the assembly of normal CE. In phase 1, the process is dependent on the membrane-bound transglutaminase, and there is deposition of soluble precursors including involucrin and small proline-rich proteins. In phase 2, which depends on both the membrane-bound and soluble transglutaminases, there is deposition of insoluble precursors such as loricrin. The sequential changes of involucrin immunoreactivity in normal CE demonstrated in the present study fit well with this model. In contrast, CE formation in psoriasis seems to remain in Eckert's phase 1, whereby CE formation is initiated precociously in the living nucleated cells. A clear explanation for the premature CE formation in psoriasis is still lacking. Increased calcium concentration in psoriatic suprabasal epidermis [30] and/or protein kinase C activation [31,32] might be relevant, because both involucrin and

Figure 5. Immunoelectron microscopy using an anti-involucrin (DH1) antibody in normal and psoriatic epidermis. *a*) In normal skin (from the neck), diffuse involucrin labeling is seen intracellularly from the granular cells (G) to the lower cornified cells (*level 2*). Labeling is greatly reduced in the more superficial cells (*levels 3,4*). In a transitional cell or early cornified cell (*level 1*), there is some labeling associated with CEs (*arrowheads*). *b*) In psoriatic skin, there is both cytoplasmic and CE-associated involucrin labeling (*arrowheads*) in the mid-epidermal layer. *c*) Most of the involucrin staining is associated with CEs (*arrowheads*) in the psoriatic stratum corneum, shown at higher magnification in *d*. d, desmosome. Bars, 0.1 μm.

the membrane-bound transglutaminase (a calcium-dependent enzyme) are induced by tumor-promoting phorbol esters in a manner dependent on protein kinase C [33,34].

REFERENCES

1. Polakowska RR, Goldsmith LA: The cell envelope and transglutaminase. In: Goldsmith LA (ed.). *Biochemistry, Physiology and Molecular Biology of the Skin.* Oxford University Press, New York, 1991, pp 168–201
2. Reichert U, Michel S, Schmidt R: The cornified envelope: a key structure of terminally differentiating keratinocytes. In: Darmon M, Blumenberg M (eds.). *Molecular Biology of the Skin. The Keratinocytes.* Academic Press, San Diego, 1993, pp 107–150
3. Hohl D, Roop D: Loricrin. In: Darmon M, Blumenberg M (eds.). *Molecular Biology of the Skin. The Keratinocytes.* Academic Press, San Diego, 1993, pp 151–179
4. Brody I: The modified plasma membranes of the transition and horny cells in normal human epidermis as revealed by electron microscopy. *Acta Derm Venereol Stockh* 49:128–138, 1969
5. Hashimoto K: Cellular envelopes of keratinized cells of the human epidermis. *Arch Klin Exp Derm* 235:374–385, 1969
6. Eckert RL, Yaffe MB, Crish JF, Murthy S, Rorke EA, Welter JF: Involucrin—structure and role in envelope assembly. *J Invest Dermatol* 100:613–617, 1993
7. Brody I: The ultrastructure of the epidermis in psoriasis vulgaris as revealed by electron microscopy. 4. Stratum corneum in parakeratosis without keratohyalin. *J Ultrastruct Res* 6:354–367, 1962
8. Mahrle G: Ultrastructure. In: Mier PD, van de Kerkhof PCM (eds.). *Textbook of Psoriasis.* Churchill Livingstone, Edinburgh, 1986, pp 113–124
9. Schaumburg-Lever G, Lever WF, Orfanos CE: Histopathology and electron microscopy. In: Roenigk HHj, Maibach HI(ed.). *Psoriasis.* Marcel Dekker, New York, 1991, pp 461–469
10. Michel S, Schmidt R, Shroot B, Reichert U: Morphological and biochemical characterization of the cornified envelopes from human epidermal keratinocytes of different origin. *J Invest Dermatol* 91:11–15, 1988
11. Martinet N, Beninati S, Nigra TP, Folk JE: N^1N^8-bis (γ-glutamyl) spermidine cross-linking in epidermal-cell envelopes. Comparison of cross-link levels in normal and psoriatic cell envelopes. *Biochem J* 271:305–308, 1990
12. Murphy GF, Flynn TC, Rice RH, Pinkus GS: Involucrin expression in normal and neoplastic human skin: a marker for keratinocyte differentiation. *J Invest Dermatol* 82:453–457, 1984
13. Bernerd BA, Asselineau D, Schaffar-Deshayes L, Yves Darman M: Abnormal sequence of expression of differentiation markers in psoriatic epidermis: inversion of two steps in the differentiation program? *J Invest Dermatol.* 90:801–805, 1988
14. Dover R, Watt FM: Measurement of the rate of epidermal terminal differentiation: expression of involucrin by S-phase keratinocytes in culture and in psoriatic plaques. *J Invest Dermatol* 89:349–352, 1987
15. Esmann J, Voorhees JJ, Fisher GJ: Increased membrane-associated transglutaminase activity in psoriasis. *Biochem Biophys Res Commun* 164:219–224, 1989
16. Eady RAJ: Transmission electron microscopy. In: Skerrow D, Skerrow CJ (eds.). *Methods in Skin Research.* John Wiley & Sons, Chichester, 1985, pp 1–36
17. Yaffe MB, Murthy S, Eckert RL: Evidence that involucrin is a covalently linked constituent of highly purified cultured keratinocyte cornified envelopes. *J Invest Dermatol* 100:3–9, 1993
18. Warhol MJ, Roth J, Lucocq JM, Pinkus GS, Rice RH: Immunoultrastructural

localization of involucrin in squamous epithelium and cultured keratinocytes. *J Histochem Cytochem* 33:141–149, 1985

19. Haftek M, Serre G, Mils V, Thivolet J: Immunocytochemical evidence for a possible role of cross-linked keratinocyte envelopes in stratum corneum cohesion. *J Histochem Cytochem* 39:1531–1538, 1991

20. Murthy S, Crish JF, Zaim TM, Eckert RL: A dual role for involucrin in the epidermis—ultrastructural localization in epidermis and hair follicle in humans and transgenic mice. *J Struct Biol* 111:68–76, 1993

21. Ishida-Yamamoto A, Hohl D, Roop DR, Iizuka H, Eady RAJ: Loricrin immunoreactivity in human skin: localization to specific granules (L-granules) in acrosyringia. *Arch Dermatol Res* 285:491–498, 1993

22. Hohl D, Olano BR, de Viragh PA, Huber M, Detrisac CJ, Schnyder UW, Roop DR: Expression patterns of loricrin in various species and tissues. *Differentiation* 54:25–34, 1993

23. Fujimoto W, Marvin KW, George MD, Celli G, Darwiche N, De Luca LM, Jetten AM: Expression of cornifin in squamous differentiating epithelial tissues, including psoriatic and retinoic acid-treated skin. *J Invest Dermatol* 101:268–274, 1993

24. Frithiof L: Ultrastructural changes in the plasma membrane in human oral epithelium. *J Ultrastruct Res* 32:1–17, 1970

25. Hashimoto K, DiBella RJ, Shklar G: Electron microscopic studies of the normal human buccal mucosa. *J Invest Dermatol* 47:512–525, 1966

26. Hashimoto K: Fine structure of horny cells of the vermilion border of the lip compared with skin. *Arch Oral Biol* 16:397–410, 1971

27. Farbman AI: Plasma membrane changes during keratinization. *Anat Rec* 156:269–282, 1966

28. Hashimoto K: Marginal band formation in keratinization. In: Hashimoto K (ed.). *Electron Microscopy of the Skin.* In Japanese. Kanehara, Tokyo, 1978, pp 19–31

29. Hohl D: Expression patterns of loricrin in dermatological disorders. *Am J Dermatopathol* 15:20–27, 1993

30. Menon GK, Elias PM: Ultrastructural localization of calcium in psoriatic and normal human epidermis. *Arch Dermatol* 127:57–63, 1991

31. Iizuka H, Takahashi H: Psoriasis, involucrin, and protein kinase C. *Int J Dermatol* 32:333–338, 1993

32. Koizumi H, Kohno Y, Osada S, Ohno S, Ohkawara A, Kuroki T: Differentiation-associated localization of nPKCη, a Ca^{++}-independent protein kinase C, in normal human skin and skin diseases. *J Invest Dermatol* 101:858–863, 1993

33. Yamanishi K, Inazawa J, Liew F-M, Nonomura K, Ariyama T, Yasuno H, Abe T, Doi H, Hirano J, Fukushima S: Structure of the gene for human transglutaminase 1. *J Biol Chem* 267:17858–17863, 1992

34. Takahashi H, Iizuka H: Analysis of the 5′-upstream promoter region of human involucrin gene: activation by 12-o-tetradecanoylphorbol-13-acetate. *J Invest Dermatol* 100:10–15, 1993