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Journal

Journal of Investigative Dermatology, 82(5)

ISSN

0022-202X

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Publication Date

1984-05-01

DOI

10.1111/1523-1747.ep12260945

Peer reviewed

Involucrin Expression in Normal and Neoplastic Human Skin: A Marker for Keratinocyte Differentiation

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Involucrin is a recently recognized structural component of mature squamous epithelial cells. We examined involucrin expression using an immunoperoxidase technique in normal skin and in a variety of epidermal hyperplasias and neoplasms to determine whether distinctive staining patterns existed within these lesions. Four patterns of reactivity were observed: diffuse intracellular staining typical of keratinocytes of the upper third of normal epidermis and epidermal hyperplasias and benign neoplasms; staining at cell borders, seen principally in benign epidermal neoplasms; patchy staining characteristic of squamous cell carcinoma *in situ*; and absence of staining in benign and neoplastic basaloid epithelium. Invasive nests of squamous cell carcinomas were negative for involucrin reactivity, whereas pseudoinvasive tongues of epithelium at the bases of keratoacanthomas were focally positive. These results suggest that immunoperoxidase staining for involucrin may be useful in distinguishing certain benign from malignant epidermal neoplasms as well as in understanding the altered maturation and kinetics of proliferative processes afflicting keratinocytes.

Involucrin is a soluble cytoplasmic protein synthesized in human squamous epithelial cells [1]. In normal epidermis, this protein is not expressed in the more basal layers [1,2], but is present in the maturing cells of the upper stratum spinosum and stratum granulosum, and subsequently appears to become incorporated into the protein envelope immediately beneath the cellular plasma membrane. This envelope is visible as the 10-nm marginal band in electron micrographs of mature squamous epithelial cells after treatment with osmium [3,4], and is stabilized by calcium-dependent transglutaminase cross-linking [5,6]. Immunologically and biochemically unrelated to keratin, involucrin is a distinctive marker for a stage in keratinocyte maturation that immediately precedes the final events of terminal differentiation [1,7-9].

Recent studies have shown that expression of involucrin is altered in neoplastic conditions, a phenomenon of potential diagnostic utility. In the uterine cervix, for example, immature squamous metaplasia and flat condylomata stain positively for involucrin by the immunoperoxidase technique, while the majority of dysplasias are negative [10]. Cultured cells from human squamous cell carcinomas and oral cavity mucosa are defective in envelope synthesis [11], and recent observations in our laboratory suggest that involucrin expression in invasive squamous cell carcinomas is markedly diminished *in vivo*.

Manuscript received August 10, 1983; accepted for publication October 20, 1983.

Supported in part by U. S. Public Health Service Grant AM 27130 from the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases.

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The purpose of this study is to document the patterns of involucrin staining in normal human epidermis and in a variety of epidermal hyperplasias, benign neoplasms, and malignant tumors. We hypothesize that if involucrin is a marker of normal keratinocyte differentiation, its expression may be altered or diminished in lesions characterized by proliferation of less differentiated basaloid or malignant cells. The observations reported herein document the patterns of immunoperoxidase staining using an anti-involucrin antiserum in normal and diseased human skin and support the concept that the expression of involucrin can be used as an indicator of keratinocyte differentiation and of malignant transformation.

MATERIALS AND METHODS

Lesions Studied

A total of 5 specimens of normal human skin and 58 specimens of epidermal hyperplasias, benign neoplasms, and malignant tumors from the surgical material of the Brigham and Women's Hospital (Boston) were examined. All specimens had been fixed in 10% neutral buffered formalin for 2-8 h (most specimens fixed for approximately 4 h), dehydrated routinely, and embedded in paraffin. All were site matched to include only biopsies of lesions from the neck, trunk, or upper arm, and all diagnoses were confirmed by a dermatopathologist (G.F.M.).

Antiserum

The primary antiserum was the rabbit antihuman involucrin described previously [1]. This antiserum is directed against involucrin that was chromatographically purified from cultured human epidermal cells and shown to be homogeneous in molecular weight and isoelectric point by gel electrophoresis. The antiserum forms a precipitin band in Ouchterlony immunodiffusion plates with less than 1 μ g of purified involucrin. Nonspecific binding to other proteins of keratinocytes or dermal fibroblasts is not detectable and no binding to involucrin is detected in preimmune serum or antiserum absorbed with purified envelopes.

Immunoperoxidase Techniques

Details of the immunoperoxidase procedure employed have been described previously [12]. Paraffin-embedded tissue sections, 4-6 μ m thick were deparaffinized in xylene and incubated with rabbit antihuman involucrin (dilution 1:1000) for 30 min, followed by sequential 30-min incubations with swine antirabbit immunoglobulin antiserum (1:30 dilution, Dakopatts, Copenhagen; US distributor: Accurate Chemical and Scientific Corp., Westbury, New York) and horseradish peroxidase rabbit anti-horseradish peroxidase (PAP) immune complexes (1:1000 dilution, Cappel Laboratories, Cochranville, Pennsylvania). Sections were then incubated with a solution containing 3,3'-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co., Milwaukee, Wisconsin; 6 mg/10 ml of 0.1 M Tris buffer at pH 7.6), and hydrogen peroxide (0.1 ml/10 ml Tris buffer) to visualize the site of antibody localization. Sections were counterstained with methyl green and mounted with Permount. Control sections utilizing either preimmune rabbit serum or immune rabbit serum that had been absorbed with purified keratinocyte envelopes were processed and in all cases were consistently negative. A section of normal skin was processed as a positive control for each case.

Characterization of Immunoreactivity

All sections were examined by 2 observers using light microscopy. Staining was characterized by location (intracellular, apparent cell

TABLE I. *Histology and involucrin reactivity in 63 biopsies of normal and lesional skin*

Lesion histology	Total No.	Site	Involucrin reactivity	Pattern
Normal	5	Back (4), neck (1)	3-4+; upper third	Intracellular, uniform
Psoriasis	6	Back (3), abdomen (3)	2-3+; upper two-thirds	Intracellular and cell borders, uniform
Lichen planus	2	Back	2-3+; all levels	Intracellular, uniform
Lupus erythematosus ^a	2	Back (1), shoulder (1)	3-4+; suprabasal	Intracellular, uniform
Seborrheic keratosis	8 ^b	Shoulder (4), neck (2), back (1), arm (1)	2-3+; upper one-sixth to two-thirds	Intracellular and cell borders, uniform
Pilar cysts	4	Back (2), shoulder (1), arm (1)	3-4+; suprabasal to all levels	Intracellular
Verruca vulgaris	5	Neck (3), shoulder (1), abdomen (1)	2-3+; suprabasal	Intracellular and cell borders, uniform
Molluscum contagiosum	6	Abdomen (3), chest (1), back (1), neck (1)	2-3+; upper two-thirds	Intracellular, uniform
Keratoacanthomas	8	Shoulder (4), back (2), arm (2)	3-4+; upper two-thirds to suprabasal; positive in pseudo-invasive lobules at base	Intracellular, uniform
Actinic keratosis	5	Shoulder (2), neck (1), back (1), arm (1)	2-3+; upper third to suprabasal	Intracellular and cell borders, uniform
Squamous cell carcinoma, in situ	6	Back (3), neck (1), shoulder (1), arm (1)	2-3+; upper one-sixth to two-thirds	Intracellular and cell borders, patchy
Squamous cell carcinoma, invasive	3	Arm (2), neck (1)	2-3+; upper half to two-thirds; negative in invasive lobules at base	Intracellular, focally patchy
Basal cell carcinoma	3	Back (1), chest (1), arm (1)	Negative in tumor; focally positive squamoid zones	Intracellular in squamoid zones only

^a Epidermis atrophic, not hyperplastic.

^b One case clinically was an epidermal nevus.

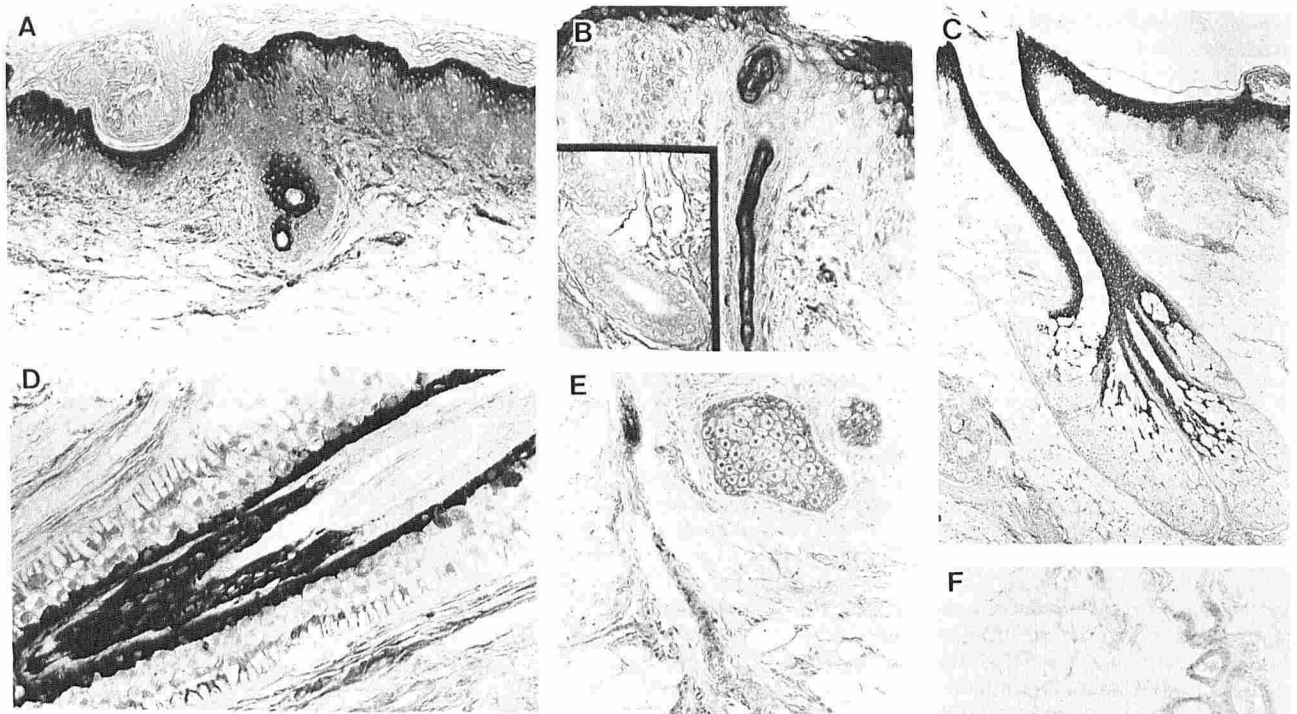


FIG 1. Normal human epidermis, formalin-fixed, paraffin section. Immunoreactivity for involucrin is localized to the upper third of the epidermis (A), the acrosyringium (A,B) and dermal eccrine duct (B); the eccrine secretory coil (B, inset) does not stain. In hair follicles (C,D) involucrin staining is restricted to the cells lining the infundibulum and sebaceous duct (C) and the layers of inner root sheath (D). Apocrine ducts originating from hair follicles stain (E, upper left-hand corner), while the secretory portion of the glands do not stain (F). (Immunoperoxidase technique; methyl green counterstain; A,E,F: $\times 86$; B,D: $\times 232$; C: $\times 38$; B, inset: $\times 370$.)

border), distribution (patchy vs diffuse), extent of staining, and involvement of epidermal appendages. In addition, the intensity of staining was judged relative to negative and positive controls on a scale ranging from 0 for negative staining to 4+ for the most intense. Positively stained cells were described as either squamoid (large, polyhydral cells) or basaloid (small, rounded to ovoid cells).

RESULTS

The lesional histology and patterns of involucrin reactivity are summarized in Table I. In normal skin (Fig 1A) approximately the upper third of the epidermis stained intracellularly

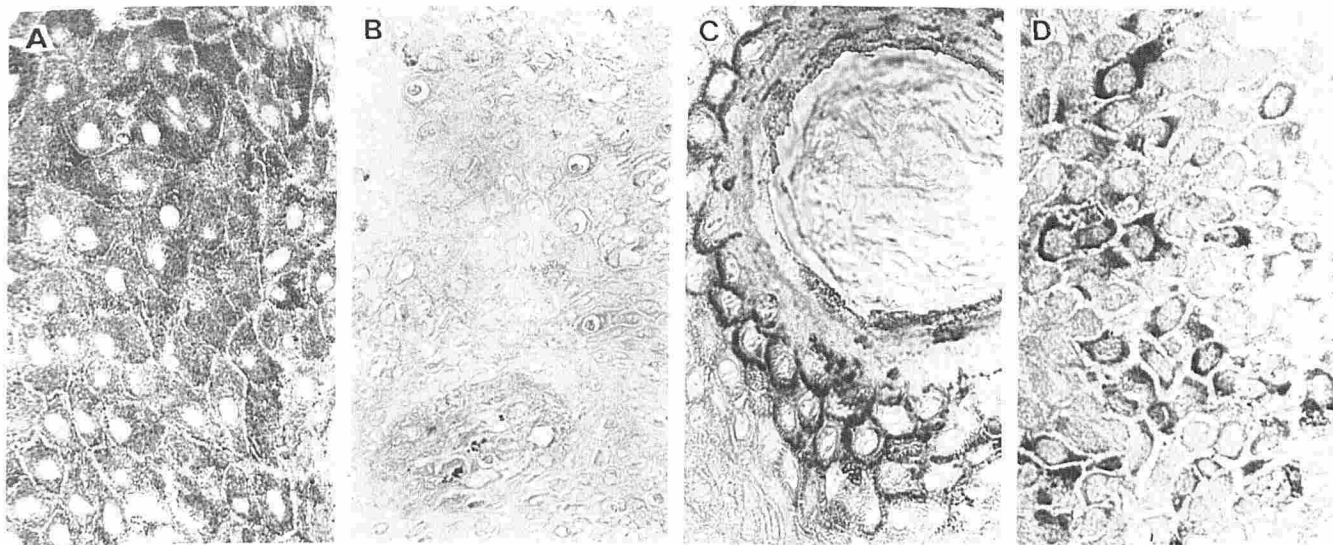


FIG 2. The four involucrin staining patterns are (A) intracellular (upper layers of normal epidermis), (B) zones of unstained cells (seborrheic keratosis), (C) staining at cell borders (seborrheic keratosis), and (D) patchy staining (squamous cell carcinoma in situ). (A,C: $\times 480$; B: $\times 370$; D: $\times 666$.)

for involucrin, whereas basal keratinocytes and immediately suprabasal layers of the stratum spinosum did not stain. The boundary between positively stained and negatively stained areas was distinct. Cells staining positively for involucrin in normal epidermal appendages included the lining epithelium of the distal portion of the dermal eccrine duct and acrosyringium, the lining cells of the apocrine duct, and the innermost lining cells of the follicular infundibulum and isthmus, as well as the cells forming the inner root sheath (Fig 1B-E). The pattern of reactivity within these structures was similar in intensity and distribution to that observed in the epidermis. Cells forming the outer root sheaths of hair follicles as well as those forming the eccrine and apocrine secretory segments were negative (Fig 1B-D,F).

Four patterns of staining were recognized. The first was homogeneous intracellular staining that characterized areas composed of cells with squamous differentiation, both in superficial layers of normal epidermis and in a variety of epidermal hyperplasias and benign neoplasms (Table I, Fig 2A). A second pattern was typical of basaloid epithelium and showed confluent zones devoid of staining. This pattern was seen in the lower layers of normal epidermis and in zones of benign epidermal neoplasms characterized by proliferation of basaloid keratinocytes, such as seborrheic keratoses (Fig 2B). A third pattern consisted of staining at cell borders without significant cytoplasmic reactivity by light microscopy (Fig 2C). This pattern accentuated the intercellular bridges and was observed in benign epidermal neoplasms, often in combination with the intracellular pattern. The fourth pattern was patchy reactivity and resembled a mosaic or "checkerboard" of stained and unstained cells (Fig 2D). This pattern was found only in squamous cell carcinomas of both in situ and invasive types. For all patterns, benign epidermal neoplasms and hyperplasias with squamoid differentiation generally showed less discrete compartmentalization of involucrin immunoreactivity than in normal skin, where the boundary between reactive and unreactive cells was abrupt. Involucrin was not detected in areas where proliferation of basaloid cells was maintained along the basement membrane zones. However, in inflammatory disorders characterized by "squamatization" of the basal layer, as in lichen planus, all layers of the epidermis stained.

Unlike normal skin, hyperplasias, and benign neoplasms, most of which had confluent zones of intense involucrin reactivity (Fig 3A-D), squamous cell carcinomas showed patchy or

negative staining for involucrin within squamoid cells (Fig 3E,F). In invasive squamous cell carcinomas, most invasive lobules at the bases of lesions did not react with antiserum for involucrin, whereas invasive lobules of histologically similar keratoacanthomas showed reactivity (Fig 3G-J). Basal cell carcinomas were uniformly negative for involucrin, except in rare foci of squamoid differentiation (Fig 3K,L).

The patterns of reactivity for involucrin within lesions did not vary within the sites examined. The nature and extent of associated inflammation appeared unrelated to involucrin expression.

DISCUSSION

These results demonstrate that involucrin expression is altered in epidermal proliferative lesions. Normal skin exhibits involucrin immunoreactivity in those cells beneath the stratum corneum that are about to undergo terminal differentiation (stratum granulosum and upper stratum spinosum). Sharp boundaries between immunoreactive cells in the upper layers of the epidermis and negative cells of the lower, less differentiated layers were observed. This reflects coordination of a normal differentiation program in which discrete epidermal compartments of involucrin expression are distinguishable. Consistent with their less mature histologic appearance, proliferative lesions formed by basaloid keratinocytes do not express detectable immunoreactivity for involucrin except in focal areas of squamoid differentiation. As a consequence, basaloid zones of benign and malignant neoplasms such as seborrheic keratoses and basal cell carcinomas are not distinguishable from each other by this criterion. By contrast, involucrin is readily detectable in benign lesions characterized by proliferation of squamoid epithelium, although it is less discretely compartmentalized than in normal skin. Abnormal patterns of involucrin reactivity are emphasized in malignant squamoid lesions where patchy immunoreactivity contrasts with the more uniform and diffuse patterns of reactivity in benign squamoid lesions. These alterations in involucrin expression reflect a lack of normal cellular coordination in preterminal differentiation in benign and malignant proliferations of squamous epithelium.

Among the squamoid lesions examined, comparison of keratoacanthomas with invasive squamous cell carcinomas provided the most striking example of differential involucrin expression. Light and electron microscopic studies of keratoacanthomas

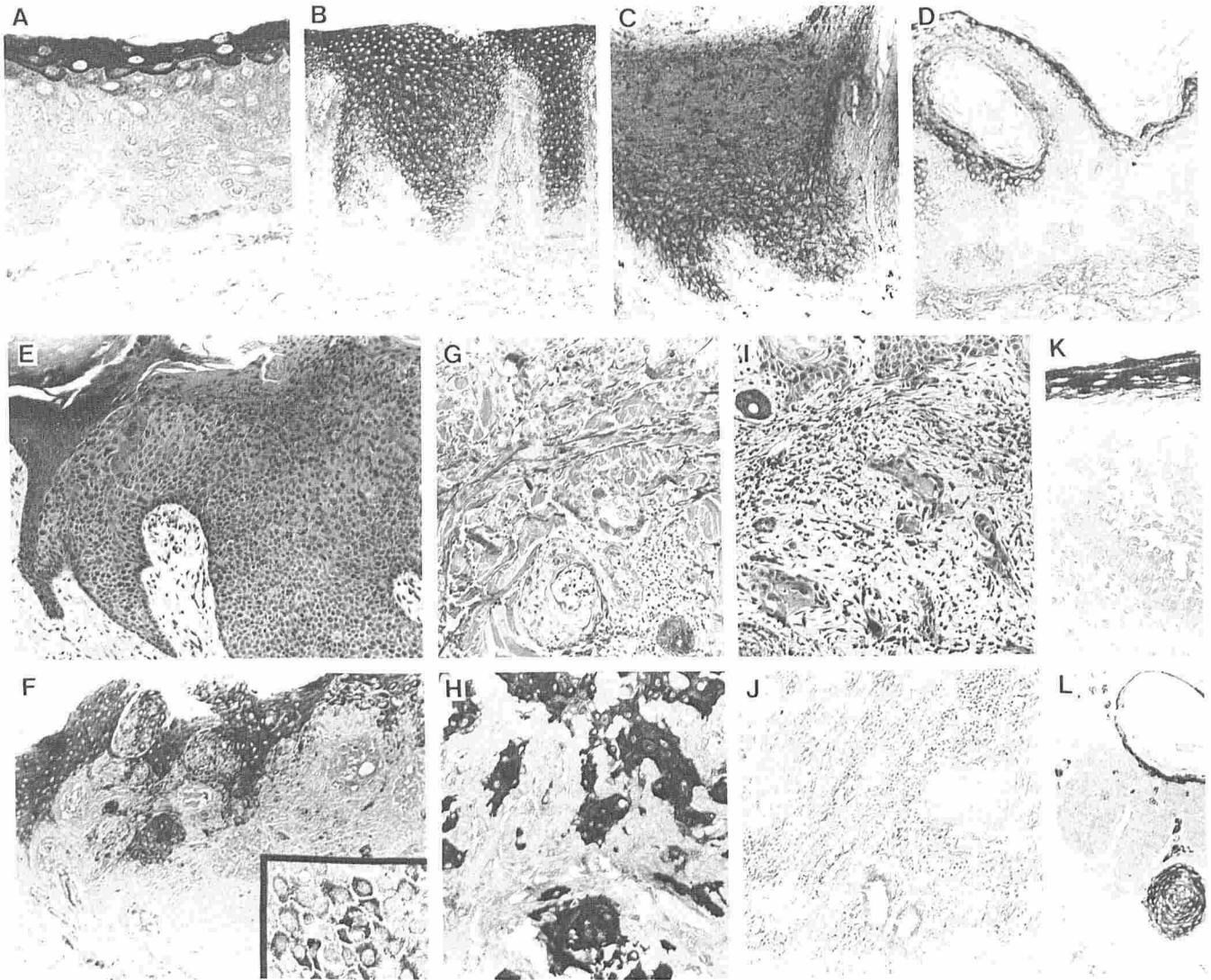


FIG 3. Involucrin staining of benign and malignant epithelial disorders. Unlike normal skin (A), epidermal hyperplasias such as psoriasis (B) and benign neoplasms such as verruca vulgaris (C) characterized by proliferation of squamoid epithelial cells, have increased immunoreactivity, whereas benign basaloid proliferations, such as seborrheic keratoses (D), have diminished immunoreactivity. Squamous cell carcinomas in situ (E, hematoxylin & eosin) show absent or patchy staining (F, inset) in zones of squamoid proliferation. Although pseudo-invasive lobules at the bases of keratoacanthomas (G, hematoxylin & eosin) stain for involucrin (H), invasive squamous cell carcinomas (I, hematoxylin & eosin) do not stain (J). Basal cell carcinomas are not immunoreactive for involucrin (K) except in focal areas of squamoid differentiation (L). (A,F, inset: $\times 370$; B,C,D,E,F,K,L: $\times 86$; G,H,I,J: $\times 132$.)

[13] and squamous cell carcinomas [14] have suggested that no single histologic feature or combination of features reliably permit differential diagnosis of these tumors. Whether differential immunoreactivity for involucrin will aid in separating these lesions requires study of a large number of cases and careful clinicopathologic follow-up.

In addition to assessment of involucrin expression in hyperplastic and neoplastic processes, it remains to be established whether deviations from the normal differentiation program in a variety of dermatoses could be reflected in a disturbed intraepidermal distribution of involucrin. Characterization of such differentiation markers may provide valuable insight not only into the histogenesis of these disorders but also into effects of treatment. The latter possibility is emphasized by the recent observation that hydrocortisone and retinyl acetate modulate involucrin levels in cultured malignant human keratinocytes [15].

Numerous markers for keratinocyte differentiation presently exist, some of which rely upon the demonstration of keratin

proteins of varying molecular weights within human epidermis [16]. Keratins are found in a variety of epithelial cell types and may be useful in characterizing specific differentiation programs [17-19]. Antiserum to involucrin, by contrast, recognizes a single protein, which is therefore a simpler marker for keratinocyte differentiation. In our experience, immunoreactivity for involucrin is also more specific for squamous epithelium than staining for keratin intermediate filament proteins [20]. These features, along with the ability of antibodies directed against involucrin to define this protein in formalin-fixed, paraffin-embedded tissues, should foster studies of involucrin expression in a variety of physiologic processes influencing squamous differentiation. Other potential envelope components may be useful also, since envelope formation may well involve more than a single protein subunit [21].

The authors gratefully acknowledge the expert technical assistance of Ms. Maureen Perry, Ms. Despina Samiotis, and Mr. Richard S. Shepard. Ms. Mary Cook provided valuable assistance in the preparation of this manuscript.

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