Morphological and Biochemical Characterization of the Cornified Envelopes from Human Epidermal Keratinocytes of Different Origin

Serge Michel, Ph.D., Rainer Schmidt, Ph.D., Braham Shroot, Ph.D., and Uwe Reichert, Ph.D.
Centre International de Recherches Dermatologiques (CIRD), Sophia Antipolis, Valbonne, France.

The formation of a cornified envelope (CE) is a major event in the terminal differentiation of epidermal cells. Nomarski contrast microscopy of the envelopes purified from different sources reveals the existence of two major, but morphologically distinct classes: the very irregularily shaped fragile type CE_f, and the polygonal rigid type CE_r. Human keratinocytes in submerged culture are only able to produce type CE_f. Specimens from healthy human epidermis contain largely type CE_r. Psoriatic scales from different patients show both types in varying proportions. Tape stripping of normal epidermis reveals that type CE_f is present in the lowermost layers of the stratum corneum and type CE_r is present in the upper layers, indicating that the two types represent a different stage of maturation.

Cyanogen bromide peptide mapping of electrophoretically purified envelopes reveals striking differences between cultured keratinocytes, normal epidermis, and psoriatic scales but also slight interindividual variations. This variability supports the view that the molecular CE composition is not strictly determined. On the other hand, no difference could be detected in the peptide maps of CE_f and CE_r obtained after tape stripping from the same healthy volunteer indicating that CE maturation within the stratum corneum does not involve the provision of qualitatively new proteins. *J Invest Dermatol 91:11–15, 1988*

The formation of a cornified envelope (CE) is one of the most characteristic features of terminally differentiating epidermal cells [2–4]. In healthy skin, the synthesis of this structure is initiated in the stratum granulosum where the cell organelles are destroyed. CE formation requires the enzyme transglutaminase, which cross-links in a Ca^{++} dependent manner pre-existing "precursor" proteins via γ-glutamyl-ϵ-lysine isopeptide bonds [5–7].

In human keratinocytes, transglutaminase exists in two forms that are probably genetically distinct: a plasma membrane associated form (TG_m) and a cytosolic form (TG_c) [8–11]. It has been shown that it is the membrane-associated enzyme that is responsible for CE formation [9–11]. Staining of histological sections of normal human skin with dansylcadaverine, a fluorescent dead-end substrate of transglutaminase, reveals the presence of membrane-associated TG activity in several layers of the epidermis reaching from the upper stratum spinosum throughout the stratum granulosum [12–14]. However, subcutaneous injection of dansylcadaverine into living human skin grafted onto nude mice demonstrates, in histologic sections, that the in vivo activity of the enzyme is restricted to one, or at most two, cell layers in the uppermost stratum granulosum [14].

Several CE precursor proteins have been detected by different techniques [15–18]. One of the major precursors in normal human keratinocytes (NHK) has been reported to be involucrin, which is especially rich in glutamine residues [19,20]. Transformed keratinocytes of the line SV-K14, however, contain very little involucrin but can nevertheless be stimulated to form envelopes that are morphologically difficult to distinguish from the envelopes synthesized by NHK in vitro [21]. Furthermore, the set of precursors used by both cell types is different. This finding and the coincidence of CE formation with cell organelle disintegration in the late stages of keratinocyte maturation led us to the hypothesis that the molecular CE composition may not be strictly determined but may vary according to the availability of potential substrate proteins for TG_m at that point in time when the enzyme becomes activated. Part of the proteins resulting from organelle destruction might be recycled as CE building blocks. We called this working concept the "dustbin hypothesis" [21]. One predication of the hypothesis is that a disturbed keratinocyte differentiation, such as that observed in several dermatoses, should be reflected in the molecular CE composition.

In the present paper, we study the morphological aspect of the envelopes obtained from cultured human keratinocytes, normal human epidermis, and psoriatic scales, and we compare the peptide patterns obtained by SDS PAGE after CE cleavage with cyanogen bromide.

MATERIALS AND METHODS

Chemicals Culture media and fetal calf serum were obtained from Flow Laboratories (McLean, VA) and GIBCO (St. Lawrence,

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Reprint request to: Dr. Uwe Reichert, Centre International de Recherches Dermatologiques (CIRD), Sophia Antipolis, F-06565 Valbonne, France.

Abbreviations:
CE: Cornified envelope
CE_f: Fragile, irregularly shaped cornified envelopes
CE_r: Rigid, polygonal cornified envelopes
EGF: Epidermal growth factor
NHK: Normal human keratinocytes
PAGE: Polyacrylamide gel electrophoresis
SDS: Sodium dodecylsulfate
TG: Transglutaminase (EC 2.3.2.13)
TG_c: Cytosolic transglutaminase
TG_m: Plasma membrane transglutaminase
Tris: Tris (hydroxymethyl) aminomethane

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MA), cyanogen bromide from Sigma (St. Louis, MO), and the chemicals used for sodium dodecylsulfate polyacrylamide electrophoresis from BIO-RAD (Richmond, CA).

Cells and Culture Conditions Normal human foreskin keratinocytes (NHK) were grown until confluence in humidified atmosphere (5% CO_2) on UV-irradiated mouse 3T3 feeder cells (ATCC line CCL92) according to the procedure of Rheinwald and Green [22] at 37°C in Dulbecco's modification of Eagle's medium and Ham's medium F12 (3:1) containing 10% fetal calf serum, 1 μM hydrocortisone, 0.1 nM cholera toxin, and 1.6 nM EGF.

Preparation of Cornified Envelopes Envelopes from cultured NHK, from psoriatic scales, and from epidermal isolates obtained by trypsination (0.25%, 1 h, 37°C), heat treatment (1 min, 60°C), or tape stripping (150–200 cm^2) were prepared. The samples were boiled under vigorous agitation for 10 min in a reaction mixture consisting of 2%(w/v) sodium dodecylsulfate (SDS) and 0.1%(w/v) dithioerythritol. The envelopes were centrifuged for 5 min at 3,500 g and suspended in fresh reaction mixture. Boiling and centrifugation were repeated four times. Then the pellet was resuspended in about the same volume of Laemmli sample buffer [62.5 mM Tris-HCl, pH 6.8, 2%(w/v) SDS, 5%(w/v) 2-mercaptoethanol, 10%(v/v) glycerol] [23] and transferred into a dialysis bag (Spectra/Por 6/132542; M_r cut off: 50,000; Spectrum Medical Industries, Los Angeles, CA). The dialysis bag was immersed in an electrophoretic tank containing Laemmli running buffer [0.2 M glycine, 25 mM Tris, 0.1%(w/v) SDS] and subjected for 24 h to 20–50 V. After washing the envelopes in running buffer and resuspending the pellet in sample buffer, electrophoretic purification was repeated once again. This procedure removed essentially all contaminating proteins as judged by the absence of migrating proteins in sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE).

Cyanogen Bromide Cleavage of Cornified Envelopes The protein content of the CE preparations was determined by the Lowry procedure after digestion of an aliquot with proteinase K (50 μg/ml). The envelopes were suspended in 70%(w/w) formic acid, and 100 mg CNBr was added per mg protein. After 24 h at room temperature the reaction was complete, leading to the solubilization of 50–70% CE protein. The mixture was diluted 1:5 with distilled water. After lyophilization, the peptides were suspended in Laemmli sample buffer [24].

Electrophoresis SDS PAGE was carried out under reducing conditions as described [23]. Fifteen percent gels were used and 100 μg of protein was routinely applied to each slot. The gels were stained with 0.2%(w/v) Coomassie Blue R 250 and destained in 50%(v/v) methanol containing 7.5%(v/v) glacial acetic acid.

RESULTS AND DISCUSSION

In a previous paper [21] we reported that serum-starved human keratinocytes of the SV-40 transformed line SV-K14 [25] can be stimulated, by treatment with the calcium ionophore A23187, to synthesize an envelope that appears under the Nomarski contrast microscope to be very similar to the envelopes formed by NHK in culture. However, the surprising result of this study was that the set of CE "precursors" used by competent SV-K14 cells differed completely from the set found in NHK [26]. Involucrin, considered a major CE constituent of NHK, could be detected in the transformed line either not at all [27] or only in trace amounts [21]. This finding led us to the hypothesis that the biochemical CE composition might not be strictly determined, but could rather depend merely on the presence of any potential substrate protein for the cross-linking enzyme when CE synthesis is initiated. As a consequence of this hypothesis, we expected differences in the protein composition of envelopes obtained from healthy individuals and from the lesional skin of patients suffering from skin disorders accompanied by aberrant keratinocyte differentiation. For this reason, we prepared envelopes (i) from several normal appearing skin specimens obtained by cosmetic plastic surgery and (ii) from psoriatic scales.

Nomarski contrast microscopy revealed striking differences between the envelopes of both sources: normal epidermis contained predominantly the polygonal rigid type CE_r (Fig 1*A*), whereas many of the envelopes from psoriatic scales were very irregularly shaped, withered, and fragile (type CE_f, Fig 1*B*). The fragile type could also be detected in normal skin samples, but only in minor

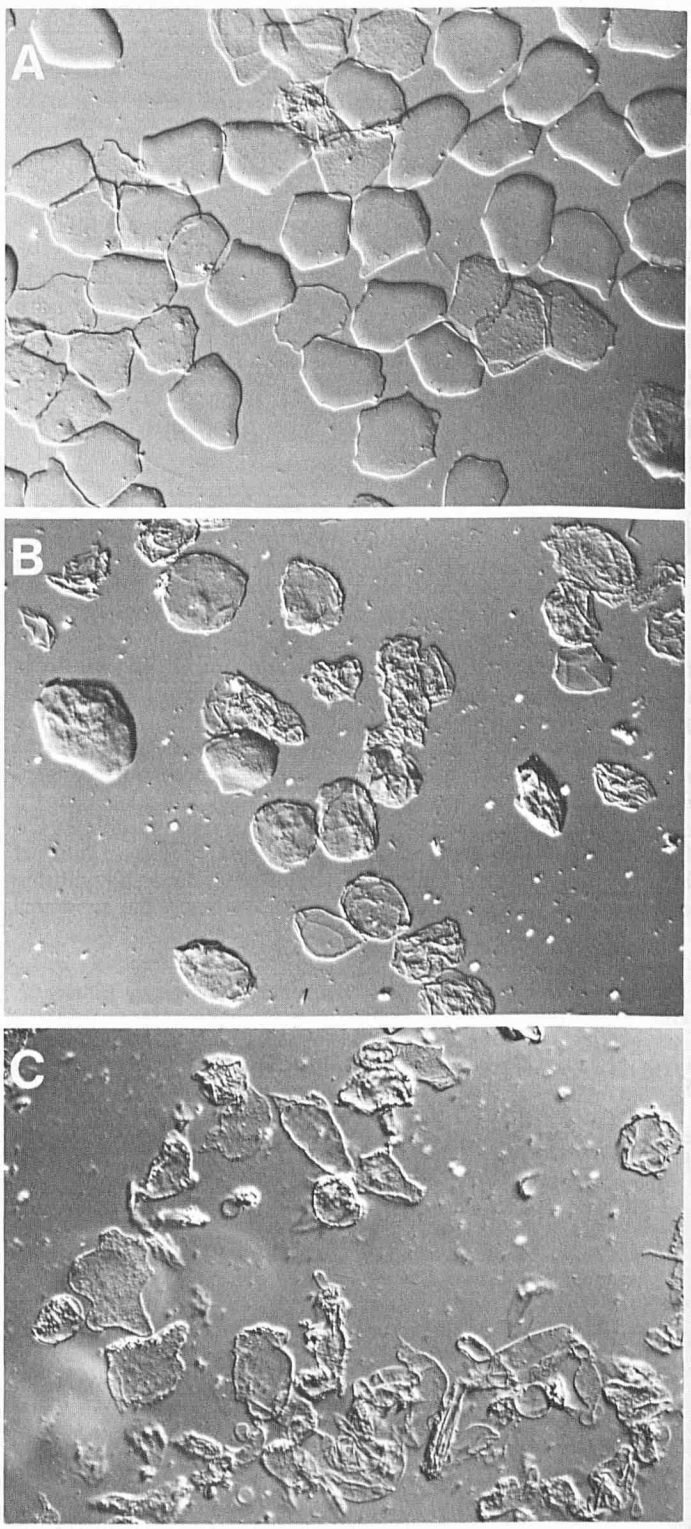

Figure 1. Nomarski contrast micrographs of the cornified envelopes obtained from normal human epidermis (*A*), psoriatic scales (*B*), and normal human keratinocytes in culture (*C*). Magnification is the same for *A-C*. Envelope diameter in *panel A* is in the order of 50 μm.

quantities. Scales from almost 40 patients suffering from stable plaque psoriasis have been analyzed so far. All of them displayed both CE types in different ratios with type CE_f; however, type CE_f was always a characteristic feature.

Because type CE_f synthesized by "psoriatic" keratinocytes closely resembled the envelopes synthesized by NHK in submerged culture (Fig 1*C*), we supposed that this type might represent an early stage in the process of CE formation. To check for this possibility we collected ten successive fractions of the horny layer from a normal skin sample by tape stripping. The first and second of five fractions were pooled, and envelopes were prepared. In parallel, histologic sections were taken to monitor the efficacy of the stripping procedure. The result of this experiment is shown in Fig 2. The panels on the left side show the histology before and after stripping. The

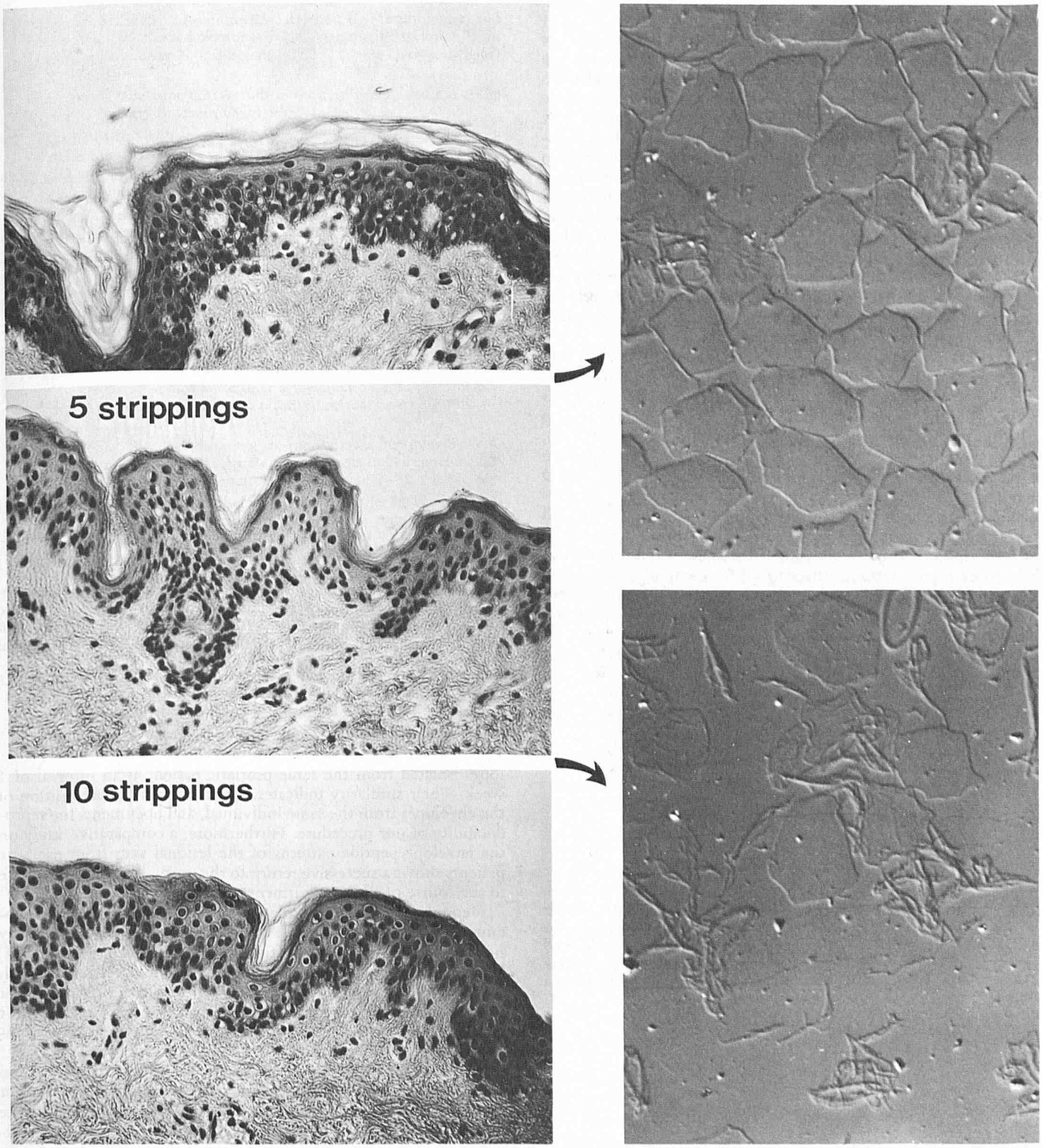

Figure 2. Histologic aspect of normal human epidermis before, after five, and after ten tape strippings *(left panels)*. Nomarski contrast micrographs of the envelopes purified from the first and second of five tape strippings *(right panels)*.

Nomarski microphotographs on the right side of the figure support our assumption that the upper stratum corneum contains almost exclusively type CE_r, whereas the lowermost horny layer is rich in type CE_f.

This result has been confirmed by performing the same type of experiment with two other skin specimens from different persons. It strongly suggests that the cornified envelope undergoes a process of maturation within the stratum corneum. This conclusion is shared by Nagae et al in a recent publication [28] which also supports our view [21] that cornified envelope composition is not strictly determined.

To find out whether the transition of envelope type CE_f into type CE_r involves the incorporation of qualitatively new proteins, we digested, by cyanogen bromide cleavage, the two CE populations obtained after tape stripping, and separated the resulting peptide mixtures by SDS PAGE. Despite some technical difficulties (low yield, interference of cellotape material with the protein determination), the almost identical peptide pattern obtained from the two envelope populations (a representative result is given in Fig. 3) does not support the assumption that qualitatively new protein entities are added within the stratum corneum to render the envelopes rigid. However, it may be possible that the ester linkage of tightly packed hydroxyacyl sphingosine with CE proteins, as suggested by Swartzendruber et al [29], is involved in CE maturation. Furthermore, it might be possible that envelope types CE_f and CE_r are related, respectively, to the stratum conjunctum and stratum disjunctum of the horny layer [30].

Cyanogen cleavage of the envelopes obtained from normal skin samples and psoriatic scales (of five individuals each) resulted in the peptide patterns of Fig 4. The difference between both groups is striking. The majority of the peptides from the "normal" group bands in three distinct regions (40, 25, and 10 kD; *lanes N1–N5*), whereas those from the "psoriatic" group are mainly found between 14 and 3 kD with three more or less pronounced bands in the 25-kD range *(lanes P1a–P5)*. Furthermore, both groups show slight interindividual variations. This is especially evident for the 40- and 25-kD range of the normal group and for the 25-kD range of the psoriatic group. It should be stressed that these variations are unlikely to be a preparation artifact based on incomplete cyanogen bromide cleavage: kinetic studies with the envelopes from different sources have shown that our experimental conditions are by far sufficient to cleave all cleavable residues.

Lanes P1a and P1b of Fig 4 show the peptide patterns of the envelopes isolated from the same psoriatic patient at an interval of 1 week. Their similarity indicates a rather constant composition of the envelopes from the same individual, and documents the reproducibility of our procedure. Furthermore, a comparative study on the envelope peptide patterns of the lesional skin from psoriatic patients shows a successive return to the normal (nonlesional) state in the course of PUVA treatment [31].

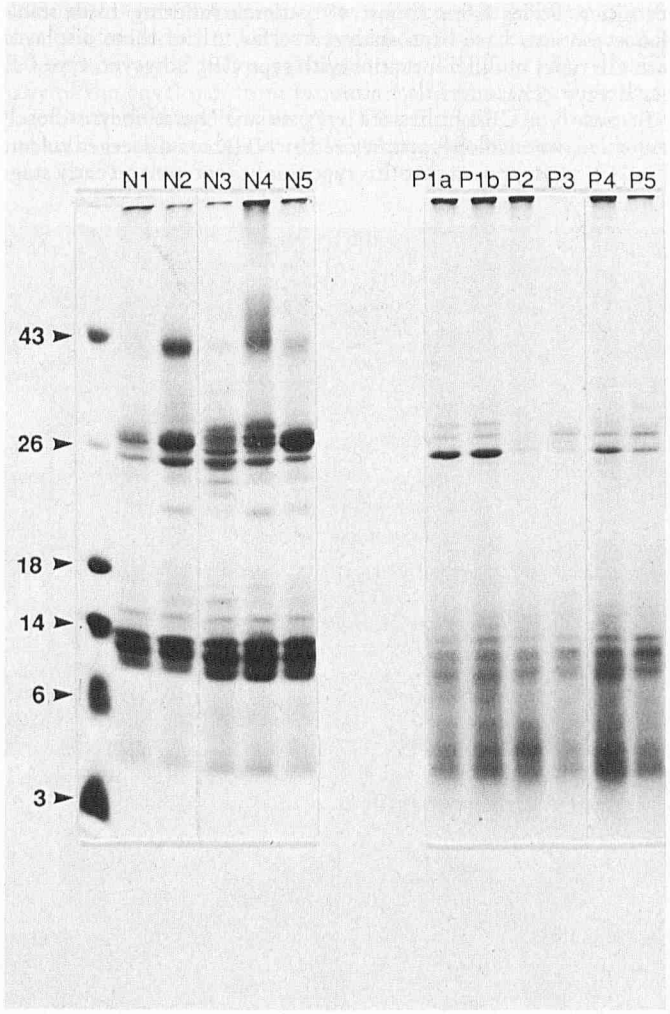

Figure 4. Cyanogen bromide peptide maps of the cornified envelopes obtained from the epidermis of five healthy individuals *(lanes N1–N5)* and from the scales of five psoriatics *(lanes P1a–P5)*. *Lanes P1a and P1b* represent the same patient from whom the envelopes were prepared at an interval of 1 wk.

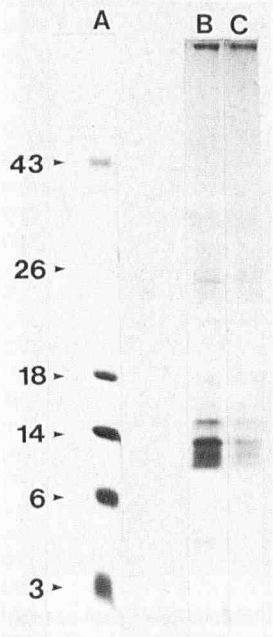

Figure 3. Cyanogen bromide peptide maps of the cornified envelopes from the first five *(lane B)* and second five *(lane C)* tape strippings of normal human epidermis. *Lane A* gives the position of molecular weight marker proteins.

A comparison of the peptide patterns from Fig 4 with those obtained after cyanogen bromide cleavage of the envelopes formed by normal and transformed keratinocytes in vitro (Fig 8 in Ref 21) shows remarkable differences: Though cultured cells share with psoriatic scales an accumulation of CE peptides in the low molecular weight range, there are some peptides that appear to be specific for either type of cultured cells and are not found under in vivo conditions.

In conclusion, the striking differences in the peptide maps of the envelopes obtained from different sources strongly favor, in our opinion, the "dustbin" concept, at least as far as the variability in the molecular envelope composition is concerned. Further experiments, however, have to prove another aspect of the hypothesis; namely, that proteins resulting from organelle destruction are also involved in envelope formation.

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