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SEPARATION OF EPIDERMAL LAYERS OF THE NEWBORN RAT

GLENN K. WALKER, PH.D.*, LEONARD SACHS, PH.D., LAURENCE A. SIBRACK, M.D., PH.D., RICHARD D. BALL, PH.D., AND I. A. BERNSTEIN, PH.D.

Cellular Chemistry Laboratory, Department of Environmental and Industrial Health, School of Public Health, The University of Michigan, Ann Arbor, Michigan, U. S. A.

A method is presented for the separation of epidermal strata by the successive elimination of either the basal or basal and spinous cells with 0.24 M NH_4Cl at pH 9.5. Histologic evidence suggests that the residual epidermal strata obtained after incubation of the skin with NH_4Cl are reproducible; hence, this technique circumvents loss of granular layer histidine-rich protein inherent with trypsin separation and provides an effective procedure for biochemical analysis of arginine-rich and lysine-rich proteins in the various differentiating epidermal cells.

Mainly four methods have been used for the separation of epidermis from underlying dermis: heating, enzymatic digestion, action of various chemicals, and mechanical separation. To date, however, no reliable procedures have been devised for separating the epidermal cell layers while circumventing the effects of trypsin on arginine-rich and lysine-rich proteins. This study introduces a reliable method for the separation of the epidermis of the newborn rat into its constituent strata after successive elution of the basal or basal and spinous cells and, hence, allows for the biochemical examination of these cellular layers and their differentiative processes.

MATERIALS AND METHODS

Skin preparation. Skins from newborn rats (1 to 2 days old) were placed (stratum corneum down) on an ice-cold watch glass and the subcutaneous fat was removed by gently scraping the dermis with a dull scalpel blade. The skins were then immersed in 0.24 M NH_4Cl, pH 9.5 (made by adding 20.4 ml HCl to 900 ml H_2O, adjusting pH with NH_4OH and bringing final volume to 1 liter), at 0°C with constant slow stirring. After 5, 15, or 30 min, the skins were removed from the NH_4Cl, placed epidermis side down on a paper towel, and the dermis was gently teased off with a forceps. In order to determine the effect of NH_4Cl (and comparatively, of trypsin) on the intact skin, skin was removed from a newborn rat, gently scraped (stratum corneum down), and small pieces were treated in 0.24 M NH_4Cl, pH 9.5, or 1.0% crude trypsin in Earle's balanced salt solution (EBSS) at 0°C for varying time periods *without* agitation and without subsequent mechanical separation. Ventral skin was utilized exclusively in all preparations.

Histologic procedures. Skin samples were fixed in neutral buffered formalin for 6 hr. After dehydration, the samples were embedded in paraffin. For cryostat sectioning, the separated epidermis was either unfixed or fixed in formalin for 6 hr, embedded in 7% agar to facilitate tissue orientation, and frozen. Sections were cut at 4 to 6 microns and were stained with hematoxylin and eosin.

For autoradiography, skins were incubated at 37°C in the dark for 1 hr in EBSS containing 10 μCi/ml [^{3}H]thymidine. Two washes for 10 min each in EBSS followed. The skins were either cut into small specimens and fixed or incubated in NH_4Cl for 5 min, after which the epidermis was separated, cut, and fixed. Autoradiograms were made with Kodak Nuclear Track Emulsion, Type NTB-2 and developed with Kodak Dektol after 2 or 3 weeks' exposure at 4°C. Autoradiograms were poststained with hematoxylin and eosin.

Biochemical analysis. Fourteen newborn rats were injected intraperitoneally with 20 μCi [^{3}H]histidine and sacrificed after 1 hr. Skins were scraped (as noted earlier) to remove subcutaneous fat and were divided into two groups. One group of 7 skins was subjected to a 5-min NH_4Cl separation; the other group was treated with 0.25% trypsin in EBSS for 15 hr at 4°C with slow stirring. The pools of epidermis were treated identically with the $HClO_4$ extraction procedure of Hoober and Bernstein [1] to obtain a histidine-rich protein.

RESULTS

Histologic examination of skins exposed to NH_4Cl reveals the following: a treatment of 5 min usually produces an even plane of separation between the basal cells and the dermis, yielding an intact epidermis. Both epidermal basal cells and

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* Present address: Department of Biology, Eastern Michigan University, Ypsilanti, Michigan 48197.

Reprint requests to: Dr. I. A. Bernstein, 1620 School of Public Health I, The University of Michigan, Ann Arbor, Michigan 48109.

Abbreviation:

EBSS: Earle's balanced salt solution.

dermal fibroblasts incorporate [^{3}H]thymidine in vitro. After incubation of labeled skin in NH_4Cl for 5 min, the dermis is removed leaving the epidermis including most of the basal cells, which exclusively contain the label (Fig. 1). Skins treated for 15 min separate between the spinous and basal cell layers (Fig. 2), whereas treatment for 30 min results in a separation between the granular and spinous cell layers (Fig. 3). Care must be taken to avoid excessive stirring and washing of the epidermis after treatment with NH_4Cl, as this may result in cells being lost from the desired skin layers.

Although the plane of cleavage is not always precisely defined, the following observations can be made regarding NH_4Cl-treated skins: an epidermal preparation made by exposure to NH_4Cl for 5 min includes basal, spinous, granular, and cornified cell layers; after 15 min only spinous, granular, and cornified cells are present in the residual strata and there are no basal cells; a preparation exposed for 30 min includes mostly granular and cornified cells with few or no spinous cells and no basal cells. Cryostat sections confirmed these results.

Skin that was treated for short time periods with NH_4Cl without agitation showed swollen, disrupted cells in the lower epidermal layers. With increased times of exposure to NH_4Cl, the number of disrupted cells increased and extended higher into the epidermis (Fig. 4). Skins treated with 1.0% trypsin for 45 min, as described above, displayed a dermis which had little or no remaining cellular structure and often showed epidermal granular layer cells with vacuoles, presumably resulting from the enzymatic digestion of keratohyaline granules (Fig. 5). Further results of histidine-protein isolation on the NH_4Cl-treated skins showed the expected high histidine radioactivity (total counts: 24×10^4); however, the same isolation on 0.25% trypsin-treated skins revealed a drastic reduction in histidine radioactivity (total counts: 9,450) where over 95% of the total counts seen in non-trypsin-treated skins were lost.

DISCUSSION

A number of techniques have been used for epidermal-dermal separation. Menschel in 1925 [2] utilized maceration in acetic acid to separate the epidermis from the dermis, and in 1927 Harris accomplished the separation by placing skins in boiling water [3]. Baumberger et al [4] in 1942 found that heating the skin at 50°C for 2 min was sufficient to produce epidermal-dermal separation in human skin and van der Leun et al [5] have recently shown a decrease in blistering time by about 13% for every 1° increase in skin temperature. A temperature of 50°C would produce a very short blistering time, and hence an easy separation. Heat, however, has one main disadvantage in that it severely denatures numerous proteins.

Medawar achieved enzymatic digestion of the epidermal-dermal junction with 0.5% trypsin in 1941 [6] and trypsin has been utilized extensively since then for epidermal separations with concentrations, times, and temperatures varying from 1.0% trypsin for 1 hr at 35°C [7] to 0.25% trypsin for 20 hr at 4°C [8] to 0.1% trypsin for 16 hr at 4°C [9]. The trypsin concentration, incubation time,

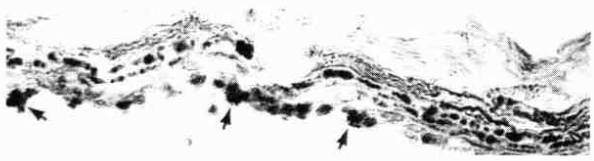

FIG. 1. Epidermis separated after 5 min of NH_4Cl treatment. This skin was labeled with [^{3}H]thymidine in vitro before the epidermal separation. Note the labeled basal cells (*arrows*) (× 340). All figures are sections of paraffin-embedded specimens.

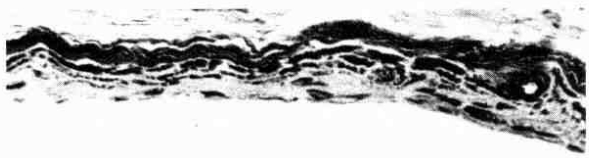

FIG. 2. Epidermis separated after 15 min of NH_4Cl treatment. Basal cells were removed and the epidermis is comprised of spinous, granular, and cornified cell layers (× 340).

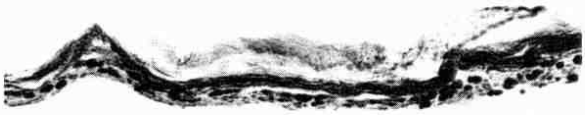

FIG. 3. Epidermis separated after 30 min of NH_4Cl treatment. Basal and most spinous cells were removed by the NH_4Cl treatment and the remaining epidermis is comprised of granular and cornified cell layers (× 340).

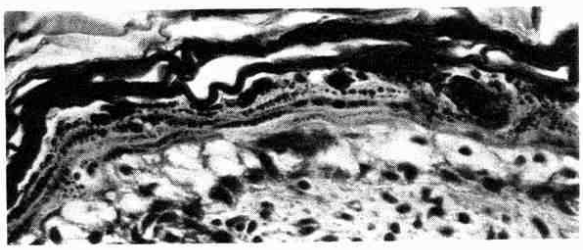

FIG. 4. Skin that has been soaked in NH_4Cl without agitation for 20 min. Note that the lower epidermal cells were swollen and disrupted by the NH_4Cl treatment. Subsequent mechanical separation of the upper epidermal cell layers is easily accomplished (× 390).

FIG. 5. Skin that has been soaked in 1% crude trypsin for 45 min at 0°C. The dermis shows little evidence of cellular structure (see Fig. 4 for comparison). *Arrows* point to vacuoles in the granular layer of the epidermis. Presumably, these vacuolated regions were once occupied by keratohyaline granules which were digested away by the trypsin treatment (× 390).

and temperature utilized in the present study are similar to those used previously by Yuspa and Harris [8] to effect epidermal separation. Unfortunately, trypsin also destroys arginine-rich proteins, such as histones, as well as the "histidine-rich protein" of the granular epidermal cells studied in this laboratory [10–12]. In fact, the action of trypsin on the keratohyaline granules (see Fig. 5, *arrows*) and on histidine incorporation may help to explain the difficulty of some workers [13,14] in obtaining high values for histidine in keratohyaline granule preparations.

Giovanella and Heidelberger [15] developed a method for the isolation of basal cells by treating mouse skin firstly with hyaluronidase and secondly with elastase. These incubations are followed with dispersal of epidermal cells (mechanically or with trypsin) in Eagle's solution and subsequent centrifugation of cells in a linear gradient of albumin in Eagle's solution. This method is said to result in a nearly pure population of basal cells.

A plethora of various chemicals has been utilized to accomplish epidermal-dermal separation. Felsher [16], in an early study, determined that 2 N solutions of a series of anions and cations detach epidermis from dermis. Approximately 10 years ago, Hoober and Bernstein [1] utilized NH_4Cl to separate the epidermis from the dermis. This technique has been altered and expanded here in order to effect the separation of epidermal strata. Although the NH_4Cl separation method appears convenient and especially appropriate for biochemical studies dealing with isolation of protein from various skin strata, it does not replace the trypsinization technique for obtaining representative population of cells from the separate layers [17]. The mechanism of disruption with NH_4Cl remains an enigma; however, a rationale for the site of action of this salt after given time periods is easier to postulate. Assuming that the cornified cell layers resist the diffusion of NH_4Cl and, therefore, that the NH_4Cl must progress through the skin from the dermis side exclusively, with increasing time, NH_4Cl diffuses further through the dermis and into the epidermis. Since the dermal-epidermal thickness (discounting the thickness of cornified cell layers) remains nearly constant in the ventral skin of the newborn rat, the plane of separation after any given time period is reproducible. Skin that was incubated in NH_4Cl lends support to the above hypothesis since, as time of treatment increases, the level of swollen cells extends higher into the epidermis.

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