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BINDING SPECIFICITY OF GUINEA PIG ANTI-α-KERATIN POLYPEPTIDE SERA ON HUMAN KERATINOCYTES: COMPARISON OF THEIR RECEPTORS WITH THOSE OF HUMAN EPIDERMAL CYTOPLASMIC ANTIBODIES

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Abstract. Experimental anti-keratin polypeptide sera (KPS) which were prepared by immunizing guinea pigs with the P1 polypeptide (molecular weight: 67 000 dalton) of α-keratin of normal human stratum corneum, were shown to react in immunofluorescence only to cell cytoplasmic antigen of the upper layers of the epidermis. No staining was detected in the basal layer. An immunolabelling performed on free epidermal cells obtained after trypsinization demonstrated by electron microscopy that receptors for KPS were tonofilaments. Minor proportions of negative cells containing tonofilaments and numerous melanosomes detected might correspond to the basal cells. An IF pattern similar to that seen with KPS was observed with some human epidermal cytoplasmic sera (ECS). However, reciprocal blocking tests performed on both rabbit lip and normal human skin with the different sera showed no inhibition. No cross-reaction was detected by immunodiffusion test between whole purified α-keratin and human ECS. In electron microscopy, the receptors for ECS appeared not to be keratin-like, but were located on a granular part of the peripheral keratinocyte cytoplasm. These findings confirmed two steps (basal and malphighian layers) in epidermal differentiation defined by antigenic markers and especially by the α-keratin component of MW 67 000 d present in normal stratum corneum.

Key words: α-Keratin; Polypeptide; Immunological properties; Epidermal cytoplasmic antibodies; Keratinocytes; Electron microscopy

Numerous analyses performed on the biochemistry and ultrastructure of α-keratin show that these fibrous proteins undergo a series of changes during keratinization which are reflected in their different physiochemical properties (3, 5, 16, 24). The protein in the Malpighian layer is solubilized by acid buffers, while that in the stratum corneum requires alkaline buffers with 6 M urea (5, 6, 16). These proteins appear heterogenous and, moreover, in the case of human epidermal proteins, differences in the polypeptide components can be observed between normals and patients with keratinization disorders (4, 15, 25, 27).

α-Keratin of normal stratum corneum is composed of three to four polypeptides of differing molecular weight and we have recently shown that the main one, of MW 67 000 dalton, was able to induce in animals specific antibodies of high titres, capable of labelling cytoplasmic antigen of keratinocytes of the spinous and granular layers of normal human epidermis, but not the basal cell layer (30). As the immunological properties of keratin components have not been thoroughly investigated (2, 27), the aim of this study was to determine, at the ultrastructural level, the exact antigenic site of the immune reaction obtained with these anti P1 polypeptide sera.

It also seemed of interest to compare these stainings with those detected with some human sera, from patients with various diseases or cancers (1, 20, 21, 28). In such human sera antibodies occur to cytoplasmic antigens of normal human epidermal cells. Nothing is known of the biochemical nature of these antigens which can be distinguished in the three types according to their location in the different layers of the skin. One is found in all keratinocytes, another only in the upper layers of the epidermis and the third only in the basal cells (10, 22). Thus, our purpose was to investigate if there is any analogy between receptors of P1 keratin polypeptide antibodies and those of human upper epidermal cytoplasmic antibodies.

MATERIALS AND METHODS

Stratum Corneum Samples

Foot callus was obtained by scraping from several individuals with no skin disorders. The material was stored at −30°C until use.

Extraction Procedures

The extraction procedure was performed according to the technique of Baden et al. (4). Briefly, squames were ground in 0.25 M sucrose in a homogenizer for 2 min at 4°C. After centrifugation at 35 000 *g* for 20 min, the pellet was homogenized in a 0.1 M sodium citrate – citric acid buffer at pH 2.65 and stirred at 4°C for 1 h. The suspension was then centrifugated at 35000 *g* for 20 min. The supernatant was discarded and the insoluble pellet was reground for 1 min at 4°C in the acid buffer and centrifugated at 35 000 *g* for 20 min. The pellet was homogenized in 6 M urea containing 0.1 M Tris, pH 9.0, and 0.1 M 2-mercaptoethanol for 24 h at room temperature under nitrogen. The suspension was centrifugated at 35000 *g* for 20 min and this last extract contained the fibrous proteins of whole stratum corneum. After extensive dialysis for 48 h against 0.025 M Tris-0-192 M glycine buffer, pH 8.3, the protein content was determined by the Lowry technique.

SDS Polyacrylamide Gel Electrophoresis

Fibrous proteins (50 μg) were dissolved in 0.0625 M Tris-HCl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue and dissociated by 2 min incubation in boiling water. They were then subjected to electrophoresis on 12% polyacrylamide slab gels prepared as described by Laemmli (13). The apparent mol. wt. of the proteins was determined according to the method of Weber & Osborn (31) using bovine serum albumin, α-globulin, ovalbumin. The first polypeptide (P1, MW: 67 000) was used for immunization.

Antisera

Keratin polypeptide sera (KPS):

Adult (400 g) female Hartley guinea pigs were immunized with the P1 keratin polypeptide according to a procedure already described (26). The animals were bled by heart puncture 8 days after the last injection. The immune sera so obtained were absorbed successively with human erythrocytes (18) and liver powder and diluted at least to 1:40 before I.F. testing.

Human epidermal cytoplasmic sera (ECS):

Various patient sera reacting with keratinocyte cytoplasm of the upper layers of the epidermis were used. They were obtained from patients with various diseases and from a patient with an IgA myeloma.

Immunofluorescence Studies (IF)

(1) On frozen sections

Indirect immunofluorescence test (IF). This test was performed on 4 μm sections of normal human skin and rabbit lip. Commercially available fluorescein isothiocyanate-labelled goat anti-guinea pig (gG (Nordic) and rabbit anti-human IgG or IgA (Behring), were used after absorption and dilution. The specificity of the IF test was shown by the absence of staining in the pre-immune guinea pig sera and in human sera negative for cytoplasmic epidermal antibodies. Immune sera were diluted in PBS at pH 7.2. Slides were viewed with a Leitz fluorescence microscope (épi-illumination orthoplan).

Reciprocal blocking tests. Specific inhibition of the cytoplasmic fluorescence reaction by KPS or by human sera with ECA (epidermal cytoplasmic antibodies) was tested on normal skin sections. Frozen sections were pre-incubated with either KPS or positive human sera for 30 min and, after being carefully washed with PBS, were subsequently incubated with the other appropriate immune sera and stained after washing with the conjugate corresponding to the second serum (rabbit anti-human IgG or IgA for human sera, or goat anti-guinea pig IgG for KPS).

(2) On human keratinocyte suspensions

10^6 cells were incubated with specific diluted antisera (1:50 for KPS and 1:20 for ECS) for 30 min. After washing, the cells were incubated with the appropriate conjugate for 30 min and washed again in PBS, mounted on glass slides and examined by fluorescence microscopy.

Immunodiffusion Test (I.D.)

Immunodiffusions tests were performed on microscope slides using 1% agarose gel in barbital buffer 0.05 M, pH 8.4. Diffusion was allowed to proceed for 24 to 48 hours at 37°C in a moist chamber.

Trypsination of Keratinocytes

Free epidermal cells were obtained from normal human skin samples after trypsinization according to the technique described by Regnier et al. (19) with slight modifications. Briefly, the samples were obtained using an electrokeratome (Castronejo type) and incubated at 37°C with a 0.5% trypsin diluted in PBS to obtain the dermal–epidermal separation. The epidermis was then washed in MEM culture medium with 10% calf serum and the keratinocytes were scattered with needles. The cell suspension was filtered through a Millipore grid and centrifugated for 10 min at 800 *g*. All cell suspensions contained more than 90% viable cells as assessed by trypan blue exclusion. The final concentration of cells was adjusted to 20×10^6 cells per ml for electron microscopic examinations.

Standard electron microscopy (EM) of keratinocyte suspensions

The cells were fixed with 2% glutaraldehyde and 1% osmium tetroxide, dehydrated and included in epoxy medium. The ultrathin sections were contrasted with uranyl acetate and lead citrate at 80 kV with a Philips EM 300 electron microscope.

Immunoenzymatic Stainings

(1) On skin sections in light microscopy

Normal skin biopsy samples were fixed with aldehyde and embedded in epoxy medium. On 2μm sections, indirect immunolabelling of keratin antigens was performed using specific sera (1:50 dilution) and immunoperoxidase conjugate (goat Po-IgG anti guinea pig IgG (Nordic) 1:50 dilution). 3-3' DAB was used to reveal conjugate peroxidase activity (12).

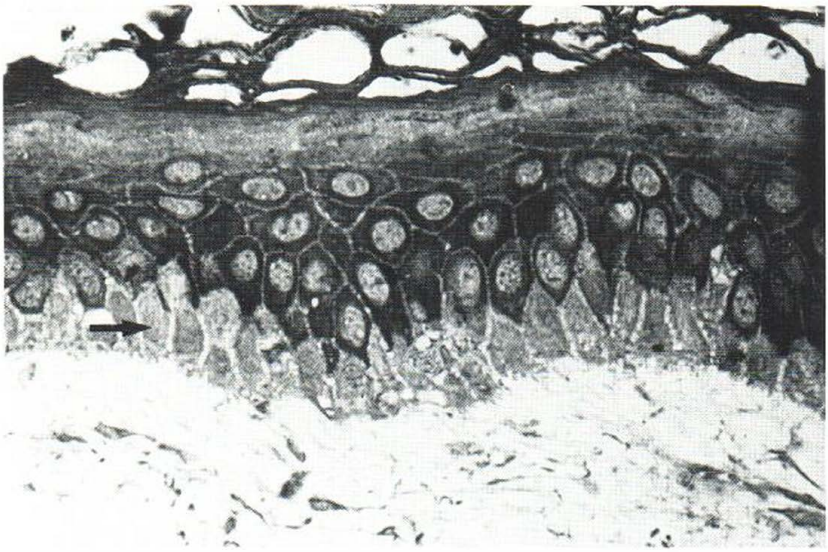


Fig. 1. Indirect immunoperoxidase labelling of the upper layer of normal epidermis with anti-P1 polypeptide sera (dilution 1 : 50) on 2 μm sections. (→) negative basal cell layer. ×605.

(2) On cell suspensions in electron microscopy

After light fixation with 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and washing with PBS, the keratinocyte suspensions were incubated with the specific antisera (anti KPS diluted 1 : 50 in PBS, anti-human ECS diluted 1 : 20 in PBS), at 37°C for 3 h. After washing, the cells were incubated with peroxidase appropriate conjugates (Po-IgG anti-guinea pig immunoglobulin (Nordic) goat and rabbit Po-Ig anti human IgG or IgA (from Institut Pasteur, Paris)) and diluted at 1 : 20 for 2 h at 37°C. After washing in PBS, the cells were fixed with 2% glutaraldehyde in cacodylate buffer. After washing in Tris-HCl buffer (pH 7.6), the peroxidase activity of the conjugate was revealed with Graham-Karnovsky medium (12). After washing in PBS, a drop of suspension was mounted on glass slide and observed by light microscopy as a control. The cells were post-fixed for 20 min with osmium tetroxide, dehydrated and embedded in epoxy medium. The cells were examined with a Philips EM 300 at 40 and 60 kV without contrast and at 80 kV with contrast as control.

RESULTS

Appearance of KPS and Human ECS by Indirect IF

On human skin and rabbit lip sections

Experimental immune sera against P1 keratin polypeptide and human sera containing upper epidermal cytoplasmic antibodies reacted to antigens present only in the upper layers of the epidermis. No staining of the basal cell layer was observed. The fluorescence was limited to the cytoplasm of epidermal cells, the nuclei were dark, the intercellular space only unstained by human ECS.

On cell suspensions

Keratin P1 polypeptide antibodies showed a bright cytoplasmic labelling which often appeared as irregular cytoplasmic spots in the majority of the cells. Human epidermal cytoplasmic antibodies revealed a diffuse, homogeneous and slightly cytoplasmic staining. In both cases, a low percentage (<10%) of small keratinocytes was unlabelled.

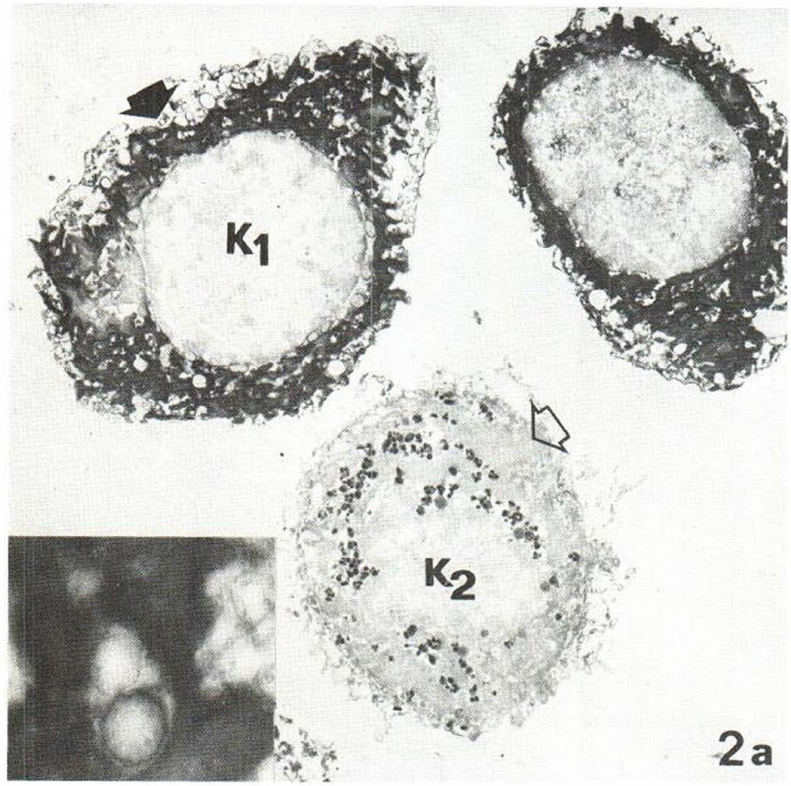
Immunoenzymatic Results

Light microscopy of skin sections

Immunoperoxidase results were similar to those obtained by indirect IF. However, the labelling on 2 μm sections allowed a more precise location of anti-P1 specificity. No staining was observed in the intercellular space or on the cytoplasmic membrane of keratinocytes (Fig. 1).

Electron microscopy (EM)

Keratin P1 polypeptide antibodies labelled specifically the cytoplasmic tonofilaments and the desmosome tonofilaments of the majority of the cells (Fig. 2*a*). At high magnification, no staining was observed on other cytoplasmic structures. After contrasting , the labelled structures correspond exactly to tonofilaments (Fig. 2*b*). A small percentage of cells remained negative and contained numerous melanosomes; after contrasting these cells also showed tonofilaments and thus might correspond to the basal layer keratinocytes (Fig. 2*b*), in ac-



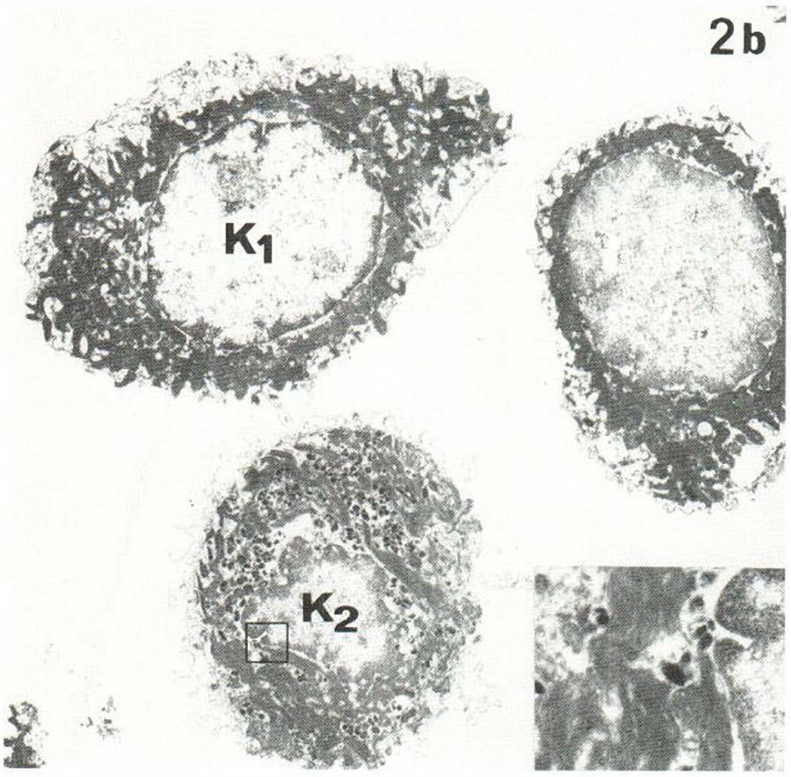


Fig. 2. (*a*) Ultrastructural aspect of the labelling of tonofilaments (*arrow*) with keratin P1 polypeptide sera. *K1*, positive cell; *K2*, negative cell. ×4600. *Inset:* detail of the labelling at high magnification. ×30000. (*b*) The same cells after contrasting, showing the keratinocytic nature of the unlabelled cell (K2). ×5000. *Inset:* detail of tonofilaments of the K2 keratinocyte. ×27600.

cordance with the large number and the repartition pattern of melanosomes in these cells.

Human epidermal cytoplasmic antibodies did not label tonofilaments but were fixed in irregular deposits in the peripheral part of the cell cytoplasm (Fig. 3*a*). However, no precision can be given as to the nature of the antigenic support (Fig. 3*b*).

Reciprocal Blocking Tests by IF Testing

Reciprocal blocking tests were performed on tissue sections of normal human skin and rabbit lip. The pre-incubation with KPS or ECS did not block the subsequent reactions with the other immune sera. Thus, no specific inhibition of the cytoplasmic reaction was obtained.

Immunodiffusion Test

Whereas anti-keratin PI polypeptide sera reacted with the whole purified stratum corneum keratin to give a precipitin line, no reaction was noted with human ECS.

DISCUSSION

We have recently shown that α-keratin polypeptides of normal stratum corneum are able to induce specific antibodies in animals (30). Using a similar biochemical analysis, several authors have reported that SDS gel electrophoresis of fibrous proteins extracted from the horny layers of normal human skin showed similar patterns, especially with a PI polypeptide of 67 000 d (5, 27). Hitherto, only the immunological properties of whole α-keratin have been studied (14) but no investigation concerning the immunological properties of α-keratin components have been reported.

Anti-cornified cell protein sera have been produced in rabbits and showed a diffuse staining of normal epidermal cells throughout the epidermis (27). We have demonstrated that the PI polypeptide of MW 67 000 d was able to induce in animals specific antibodies labelling only the keratinocyte cytoplasm of the upper layers of the epidermis. The immunological properties of such a polypeptide exhibited an important difference between the basal cells and the others located above, thus confirming numerous studies which have already reported differences in morphology and biochemistry between these two cell populations (11, 17). Consequently, the polypeptide of MW 67 000 d might be considered as a specific cellular marker in the differentiative pathway of keratinization. Moreover, immunoelectron microscopy allows us to define accurately the nature of the antigenic sites of KPS and to compare them with those recognized in some human ECS.

Indirect IF studies are not sufficiently precise to give valuable information on the nature of these antigens. However, they did allow us to study the antigen histological location and the specificity of the antisera by means of reciprocal blocking tests.

By the immunoperoxidase procedure, the same patterns can be obtained on frozen fixed tissue sections in light microscopy. The same labelling has been performed in EM on skin sections mounted on glass slides (23). However, this method does not allow us to obtain really valuable and reproducible results, as the immunological reagent penetration was inadequate.

By using isolated keratinocytes, it was possible to obtain a better immunological staining of the cytoplasmic antigens. Ther keratinocyte trypsinization allowed us to perform an immunocytological study on viable cells such as can be seen after ultrastructural control.

The EM stainings revealed by KPS and human ECS are related to antigens differing according to their location and structure. These results demonstrated that antigenic support of human ECS did not correspond to tonofilaments.

Keratin antigens are easily defined ultrastructurally but the nature of cytoplasmic antigens detected by human sera cannot be established with precision.

The negative basal cells are characterized by the absence of these antigens and especially the PI polypeptide keratin even though these cells contain tonofilaments similar to those of Malpighian cells. In contrast, tonofilaments bound to the Malpighian layer acquired the PI specificity (Fig. 2*a*).

Recently, Breathnach pointed out that basal tonofilaments are identical with those of the horny cells (7). However, Brody's studies described morphological and histochemical differences between them and suggested that the horny cell filaments are not identical with those of the basal cells (8, 9). The fibrillar differentiation suggested by Brody's theory on the basis of morphological observations seems to be confirmed by the immunological properties of the PI polypeptide. In some cases, the cell differentiation is characterized by the acquisition of specific antigenic markers and

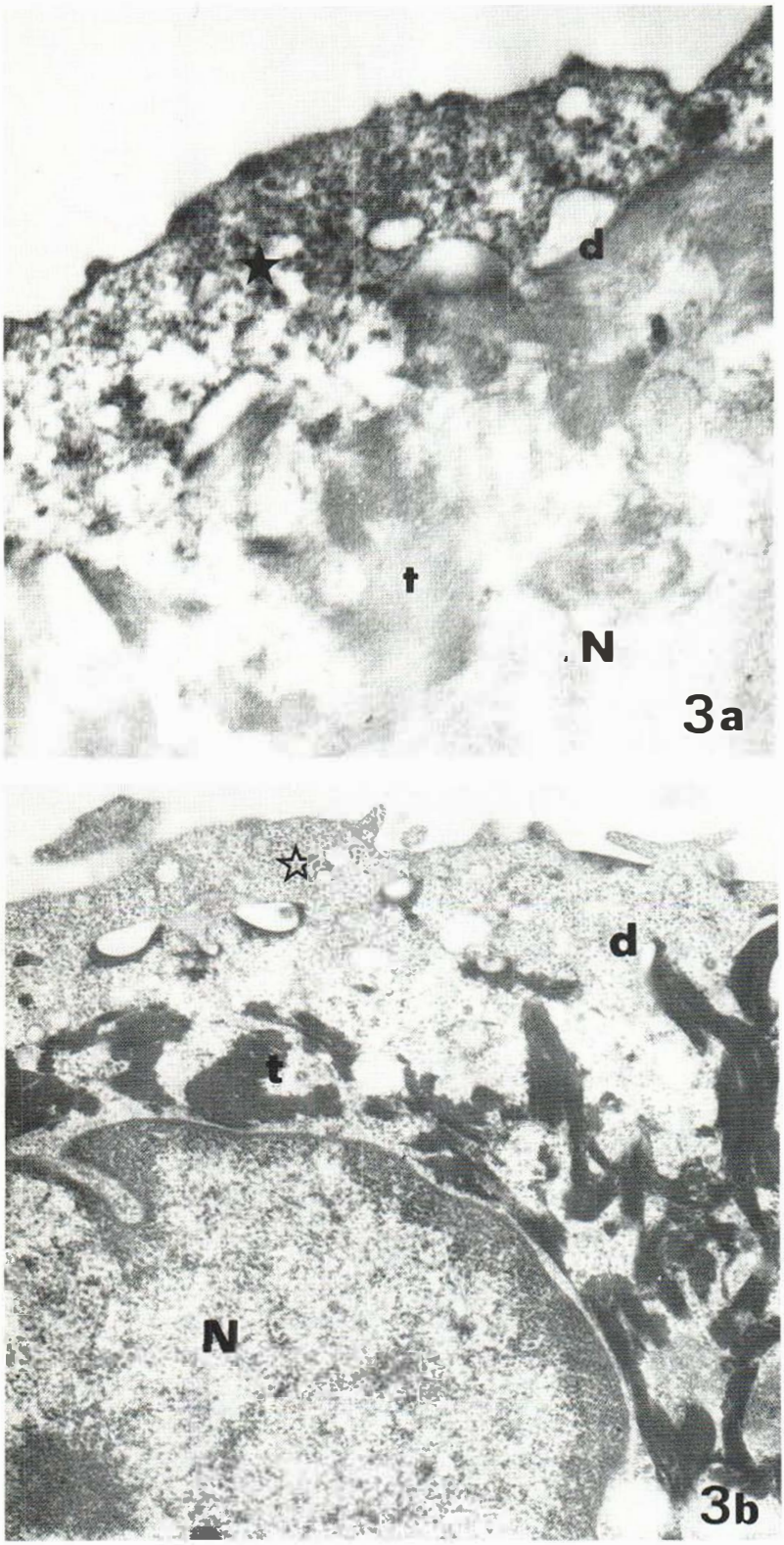


Fig. 3. (*a*) Ultrastructural aspect of the labelling (★) obtained with human epidermal cytoplasmic antibodies. No dense deposits on tonofilaments (*t*). *d*, desmosomes; *N*, nucleus. ×34 000. (*b*) Ultrastructural picture of keratinocyte in standard EM showing the granular aspect of the peripheral cytoplasmic zone (☆) labelled with ECS. *d*, desmosomes; *N*, nucleus; *t*, tonofilaments. ×24 000.

functional properties, as has been demonstrated for example for the thymus-dependent lymphocyte population (19). With keratinocytes, we may assume that sequential stages of differentiation can be defined by antigenic markers directly related to the specific functional properties of epidermis which is the keratinization process.

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REFERENCES

1. Abel, E. A. & Bystryn, J. C.: Epidermal cytoplasmic antibodies: incidence and type in normal persons and patients with melanoma. J Invest Dermatol *66:* 44, 1976.
2. Baden, H. P., Goldsmith, L. A. & Gifford, A. M.: Immunologic properties of the alpha fibrous protein of human epidermis. Acta Dermatovener (Stockholm) *51:* 327, 1971.
3. Baden, H. P., Lee, L. D. & Kubilus, J.: The fibrous proteins of stratum corneum. J Invest Dermatol *67:* 573, 1976.
4. Baden, H. P., McGilvray, M., Cheng, C. K., Lee, L. D. & Kubilus, J.: The keratin polypeptides of psoriatic epidermis J Invest Dermatol *70:* 294, 1978.
5. Baden, H. P. & Lee, L. D.: Fibrous protein of human epidermis. J Invest Dermatol *71:* 148, 1978.
6. Baynes, J., Levine, M., McLeod, A. & Wilkinson, A.: Precursor keratin protein from human epidermis. Br J Dermatol *98:* 165, 1978.
7. Breathnach, A. S.: Aspects of epidermal ultrastructure. J Invest Dermatol *65:* 2, 1975.
8. Brody, I.: The ultrastructure of tonofilaments in the keratinization process of normal human epidermis. J Ultrastruct Res *4:* 264, 1960.
9. Brody, I. : Variations in the differentiation of the fibrils in the normal human stratum corneum as revealed by electron microscopy. J Ultrastruct Res *30:* 601, 1970.
10. Bystryn, J. C., Abel, E. A. & Weidman, A.: Antibodies against the cytoplasm of human epidermal cells. Arch Dermatol *108:* 241, 1973.
11. Fukuyama, K., Nakamura, T. & Bernstein, I.: Differentially localized incorporation of amino acids in the newborn rat. Anat Rec *152:* 525, 1965.
12. Graham, R. C. & Karnowsky, M. J.: The early stages of adsorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J Histochem Cytochem *14:* 291, 1966.
13. Laemmli, U. K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *277:* 680, 1970.
14. Lee, L. D., Baden, H. P., Kubilus, J. & Fleming, B. C.: Immunology of epidermal fibrous proteins. J Invest Dermatol *67:* 521, 1976.
15. Levine, M. & McLeod, A.: Fibrous proteins of normal and abnormal human epidermis. Br J Dermatol *100:* 401, 1979.
16. Matoltsy, A. G.: Desmosomes, filaments, and keratohyaline granules: their role in the stabilization and keratinization of the epidermis. J Invest Dermatol *65:* 127, 1975.
17. Moore, J. T. & Karasek, M. A.: Isolation and properties of a germinative and a non germinative cell population from post embryonic mouse, rabbit and human epidermis. J Invest Dermatol *56:* 318, 1971.
18. Olson, K., Biberfeld, G. & Fagraeus, A.: Blood group antibodies as a source of error in the diagnosis of pemphigous by indirect immunofluorescence. Acta Dermatovener (Stockholm) *52:* 389, 1972.
19. Regnier, M., Delescluse, C. & Prunieras, M.: Studies on guinea pig skin cell cultures. I. Separate cultures of keratinocytes and dermal fibrocytes. Acta Dermatovener (Stockholm) *53:* 241, 1973.
20. Saurat, J. H., Didierjean, L., Gluckman, E., Lauraine-Pujade, M. D. & Puissant, A.: Studies on epidermal cytoplasmic antibodies. J Invest Dermatol *68:* 245, 1977.
21. Saurat, J. H, Didierjean, L., Beucher, F. & Gluckman, E.: Immunofluorescent tracing of cytoplasmic components involved in keratinocyte differentiation. Br J Dermatol *98:* 155, 1978.
22. Saurat, J. H., Didierjean, L., Galoppin, L. & Gluckman, E.: Antibodies with affinity to the cytoplasm of keratinocytes produced after bone marrow transplantation in man. A review. *In* "Cutaneous Immunopahology". Publication INSERM (ed. J. Thivolet & D. Schmitt). *80:* 77, 1978.
23. Schmitt, D.: Ultrastructural immunohistochemical technique applied on skin sections mounted on glass slides. *In* "Cutaneous Immunopathology". Publication INSERM (ed. J. Thivolet & D. Schmitt), *80:* 23, 1978.
24. Skerrow, D., Matoltsy, A. G. & Matoltsy, M. N.: Isolation and characterization of epidermal prekeratin. Biochim Biophys Acta *317:* 303, 1973.
25. Skerrow, D. & Hunter, I.: Protein modifications during the keratinisation of normal and psoriatic human epidermis (BBA 38051). Biochem Biophys Acta *537:* 474, 1978.
26. Staquet, M. J., Viac, J. & Thivolet, J.: Antigenic relationships between polypeptides derived from plantar and hand wart viruses. J Gen Virol *43:* 713, 1979.
27. Thaler, M. P., Fukuyama, K., Inoue, N., Cram, D. L. & Epstein, W. L.: Two tris urea mercapto-ethanol extractable polypeptides found uniquely in scales of patients with psoriasis. J Invest Dermatol *70:* 38, 1978.
28. Thivolet, J. & Beyvin, A. J.: Recherche par immunofluorescence d'autoanticorps sériques vis à vis des constituants de l'épiderme chez les brûlés. Experientia *24:* 945, 1968.
29. Touraine, J. L., Hadden, J. W. & Good, R. A.: Se-

quential stage of human T lymphocyte differentiation. Proc Natl Acad Sci USA *74:* 3414, 1977.
30. Viac, J., Staquet, M. J., Goujon, C. & Thivolet, J.: Experimental production of antibodies against stratum corneum keratin polypeptides. Arch Dermatol (accepted for publication).
31. Weber, K. & Osborn, M.: The reliability of molecular weight determinations by dodecyl sulfate polyacrylamide gel electrophoresis. J Biol Chem *244:* 4406, 1969.

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