Acta Dermatovener (Stockholm 57: 289–296, 1977

NEW STAINING TECHNIQUES FOR THE LANGERHANS CELL

Lennart Juhlin and Walter B. Shelley

From the Department of Dermatology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Abstract. Three new techniques are described for staining the Langerhans cell in whole mounts of fresh human and guinea pig epidermis. These employ paraphenylenediamine, gold sodium thiomalate and cobalt chloride, respectively, and require appropriate epidermal separation with EDTA, ammonium thiocyanate or sodium bromide. Used in conjunction with a modified adenosine triphosphatase stain, these techniques provide greater capability for observing the Langerhans cell in disease states than can be achieved by any single stain. A combined stain with adenosine triphosphate and gold is also described.

Key words: Langerhans cell: Lysozyme separation: ATPase: Paraphenylenediamine: Cobalt: Gold sodium malate

The Langerhans cell has been a strangely elusive cell ever since its initial sighting in 1868. It never had appeared in routine haematoxylin-eosin sections. Even when stained with gold chloride or osmium zinc iodide its dendritic structure blended in with the melanocyte lineage. Only recently has the cell yielded the secret of its distinctive fine structure and remarkable enzyme content (2, 7, 3, 8, 13).

The Langerhans cell has eluded not only routine histologic definition but also functional assessment. Attempts have been made to assign the cell a role in sensory perception, pigmentation, keratinization or any disease state, but an air of mystery still surrounds the cell. However, there is growing evidence in favor of a role for Langerhans cells in contact allergic hypersensitivity, as recently reviewed by Silberberg et al. (11). Moreover, work in our laboratory demonstrating that the cell may be an epidermal contact allergen trap (10), forming a unique reticulo-epithelial system, makes it essential to develop better methods for its visualization in a variety of conditions. The present report details new methods we have developed for staining this elusive cell.

MATERIAL AND METHODS

I. *Specimens*

The majority of studies were done on the ears of male albino guinea pigs (300 to 400 g). The ears were excised immediately after the animal was killed by cervical dislocation. Skin was also taken from other areas such as the back, foot pads and scrotum. It was washed, the hair removed and stratum corneum scraped clean to glistening by means of a razor blade.

"Epidermal" biopsy specimens (1 cm^2) were taken from the normal skin of white male and female human subjects of different age groups. Anesthesia was achieved by injection subepidermally of 1 ml of a 2% solution of lidocaine (Xylocaine®). The specimen was obtained by a gentle sawing cut with a sterile half-blade (a new Gillette Super-Blue Blade®), kept superficial and parallel with the surface, which is held taut during procedure.

The epidermis was separated from the dermis by a variety of methods outlined in Table I. These permitted gentle manual lifting off of a full sheet of epidermis by means of Swiss Forceps No. 5.

II. *Solutions*

In addition to the separation fluids prepared as in Table I, the following were used in the staining techniques to be described:

"Fixatives"

Cacodylate–Formaldehyde
- 6.85 g sucrose
- 10 ml 40% formaldehyde
- 40 ml 0.2 M (i.e. 3.2%) cacodylic acid (Sigma)
- 50 ml distilled water

pH 4–Formaldehyde
- 1 g pH 4 buffer salt mix (potassium phthalate–tartaric acid) (Harleco, Hartman-Leddon Co., Philadelphia, Pa.)
- 100 ml cacodylate–formaldehyde solution (see above)

Table I. *Epidermal separation techniques (5, 1)*

Separation fluid	Composition	Time (min)	Temperature
EDTA	NaCl 6.83 g KCl 0.20 g Na_2HPO_4 1.15 g KH_2PO_4 0.20 g Phenol red 1% 0.12 ml Na_4EDTA (ethylenediamine tetra acetic acid tetrasodium salt) 7.60 g in liter. Adjust pH to 7.2	120	37°C
Ammonium thiocyanate	0.5 mole in 0.1 M phosphate buffer, pH 6.8	20	37°C
Sodium bromide	1 mole in 0.1 M phosphate buffer, pH 6.8	60	37°C
Lysozyme	0.05 mg/ml 0.1 M phosphate buffer, pH 6.8	90	37°C
Trypsin	1/10000 aqueous 1× crystallized 50% $MgSO_4$ (Worthington) in phosphate buffer, pH 7.4	45	37°C
Alkali	1% Borate–carbonate buffer, pH 11.0 (Harleco)	30	37°C
Heat	Slide warmer	2	49.2°C
Ammonium	1 M ammonium hydroxide (7 parts) 1 M ammonium chloride (3 parts) Final pH 9.6	20	25°C

Stains

ATP–Lead:

Stock ATP
- 50 mg adenosine 5'triphosphate disodium salt (Sigma)
- 5 g glucose
- 50 ml distilled water
- 40 ml Trismal buffer (see below)
- 10 ml 0.1 M (i.e. 1.2%) $MgSO_4 \cdot 7\ H_2O$ in distilled water

Trismal buffer
- 12.1 g Tris buffer salt (Harleco)
- 11.6 g maleic acid (Sigma)
- 4.0 g sodium hydroxide
- 400 ml distilled water

Final ATP–Pb staining solution
- 2.7 ml stock ATP solution
- 0.3 ml 2% lead nitrate in distilled water

Cobalt
- 0.3 ml 5% cobalt chloride–hexahydrate
- 2.7 ml metal-free Timm's stock solution (see below)

Gold
- 0.6 ml gold sodium thiomalate (disodium mercaptosuccinate gold, Myochrysine®, Merck), 5% aqueous solution (containing 0.5% benzyl alcohol)
- 2.4 ml pH 3.0 buffer (1% K phthalate–tartaric acid powder, Harleco)

Nickel
- 0.3 ml 5% nickel chloride hexahydrate
- 2.7 ml metal-free Timm's stock solution (see below)

Paraphenylenediamine
- 0.5% in pH 11.0 buffer (1% borate–carbonate salt, Harleco)

Developers

Ammonium sulfide
- 5 ml 22.3% ammonium sulfide solution (Baker)
- 95 ml distilled H_2O
- Must prepare fresh if any color change or precipitate appears.

Hydrogen peroxide
- 3% (Baker)

Silver Timm solution
- 0.3 ml 5% silver nitrate in distilled water
- 2.7 ml metal free stock Timm's solution (see below)

Timm stock (metal-free)
- 30 g acacia (gum arabic)
- 10 g sucrose
- 0.43 g citric acid
- 0.17 g hydroquinone
- 100 ml distilled water
- Use hot water and mix well in preparing.

Rinse

Distilled water

Mounting medium

Glycerine jelly (Kaiser)

III. *Staining methods*

Adenosine triphosphatase. EDTA-separated epidermis is placed immediately in saline solution for 30 minutes at room temperature. It is then immersed in cacodylate–formaldehyde solution for 20 minutes at 4°C. Rinse. Immerse in ATP–Pb solution at 37°C for 20 minutes (guinea pigs) or 60 minutes (human). After rinsing, it is immersed in ammonium sulfide solution for 20 minutes at room temperature. After rinsing, mount dermal (dark) side up in glycerine jelly.

Gold. Ammonium thiocyanate separated epidermis is immersed in saline for 30 minutes at room temperature. This is followed by immersion in Myochrysine® solution for 60 minutes at 37°C. Rinse. Immerse in ammonium sulfide solution for 20 minutes at room temperature. Rinse. Immerse in cacodylate–formaldehyde solution for 30 minutes at 4°C. Rinse. Immerse in silver Timm's solution for 6 minutes at 66°C. Rinse. Mount.

Paraphenylenediamine. Ammonium thiocyanate separated epidermis is placed immediately in saline solution

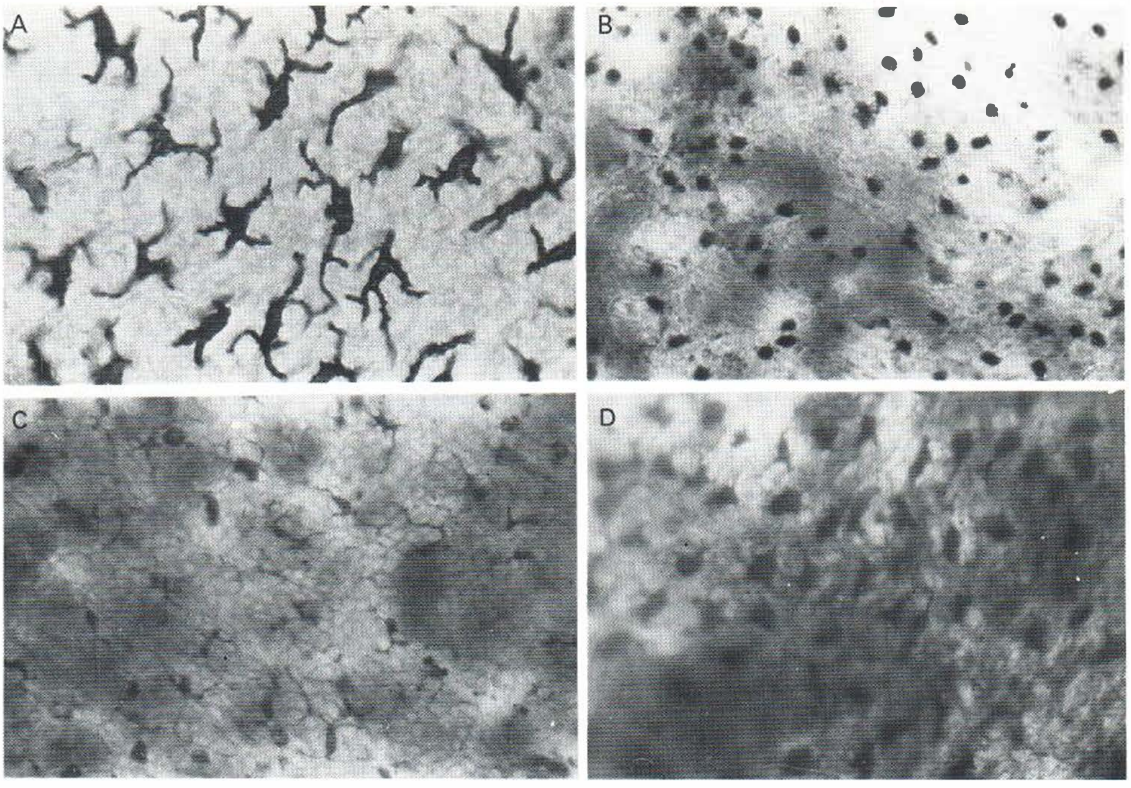

Fig. 1. Langerhans cells in epidermal sheets from guinea pig ears, employing techniques described in text. (A) ATPase stain, (B) Gold (Myochrysin), (C) Paraphenylenediamine, (D) Cobalt. Surface view, ×390 (1A, 1D); ×245 (1B, 1C).

for 30 minutes at room temperature. It is then kept in cacodylate–formaldehyde solution for 60 minutes at 4°C. Rinse and treat in paraphenylenediamine solution for 60 minutes at 37°C. Rinse. Immerse in hydrogen peroxide solution for 10 minutes at room temperature. Rinse with care and mount. If bubbles interfere, remount. Read within short time.

Table II. *Langerhans cell staining results. Guinea pig ear epidermis*

++++=excellent best, +++=good, ++=fair, +=poor, 0=no stain
Grading 0 to 4+

Separation method	ATP	Gold	Paraphenylenediamine	Cobalt
EDTA	++++	+	++	+
Thiocyanate	+++	++++	++++	+++
Sodium bromide	++	+++	++	++++
Lysozyme	+++	0	+++	+
Trypsin	++	0	0	+
Alkali	+++	●	+++	++
Heat	0	++	+++	0
Ammonium salts	0	0	0	0

Cobalt. Sodium bromide separated epidermis is placed immediately in saline solution for 30 minutes at room temperature. It is then immersed in pH 4 formaldehyde solution for 60 minutes at 4°C. Rinse. Immerse in ammonium sulfide for 60 minutes at room temperature. Rinse. Treat in cobalt–Timm's solution for 20 minutes at 66°C. Rinse and mount.

For nickel stain, employ same procedure, but substitute nickel Timm's solution for cobalt Timm's solution.

Combined ATP–gold stain. Ammonium thiocyanate separated epidermis is kept in saline solution for 30 minutes at room temperature. It is then fixed and stained as described above for ATP. After the ammonium sulfide and rinsing steps, it is immersed in Myochrysine® solution for 60 minutes at 37°C. Rinse. Immerse in ammonium sulfide solution for 20 minutes at room temperature. Rinse. Immerse in silver Timm's solution for 5 minutes at 66°C. Rinse. Mount.

IV. *Cross-sections*

The stained sheets of epidermis could be hand cut transversely with a razor blade or embedded in paraffin, sectioned with a regular microtome and mounted in the usual fashion.

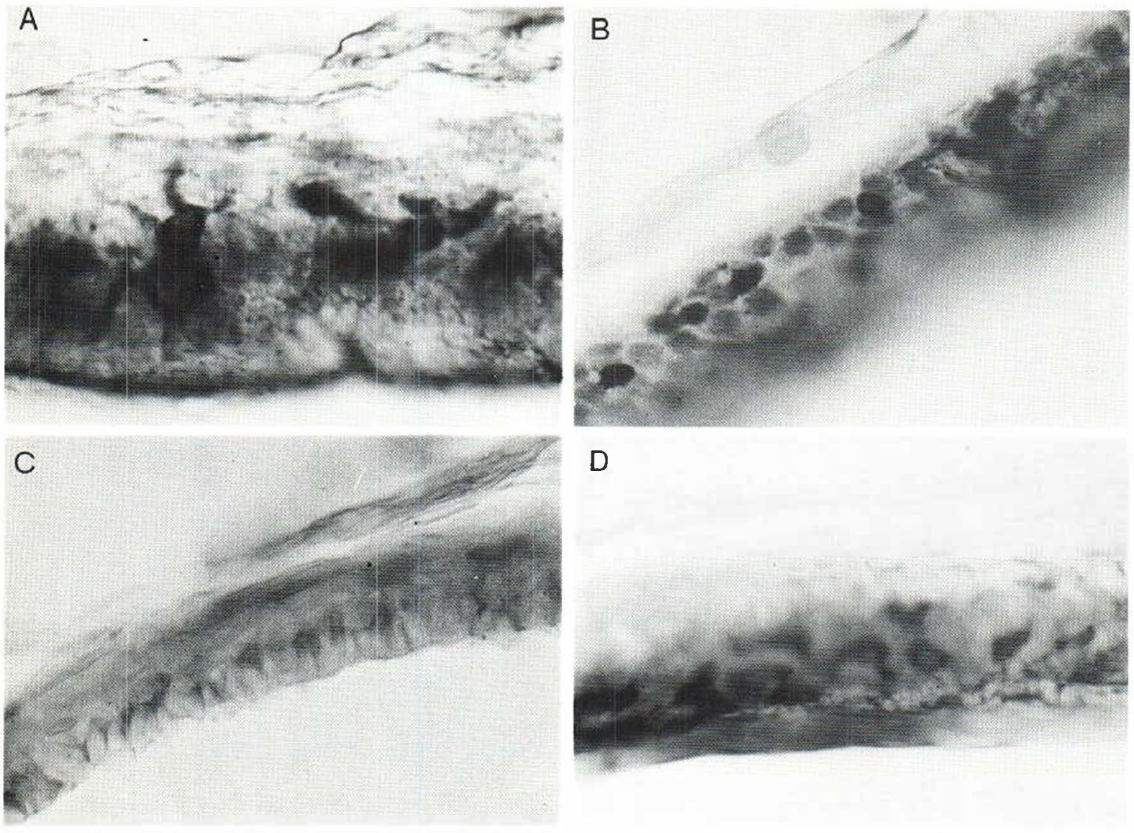

Fig. 2. Hand razor blade cross section of epidermal sheets shown in Fig. 1, showing Langerhans cell in mid-zone of epidermis. (A) ATPase stain, (B) Gold (Myochrysin), (C) Paraphenylenediamine, (D) Cobalt.

RESULTS AND DISCUSSION

With the techniques we have described, the Langerhans cells can be well visualized in normal human and guinea pig skin (Fig. 1). By staining the entire epidermal sheet and viewing it en face, one is assured of a panoramic topographic view of the cell inter-relationships. The classical cross section (Fig. 2) does enable one to identify the cells as high level dendritic cells but, since one can only focus on a few cells, it sharply restricts one's sense of the reticulo-epithelial nature of this Langerhans network.

As Table II makes evident, the best staining is achieved only when the epidermal tissue is treated in a manner appropriate for the specific stain being done. As an example, the use of ammonium hydroxide as a separative completely prevents subsequent staining. Moreover, simply allowing the properly separated epidermis to remain in saline too long or in distilled water can seriously interfere with certain stains. Despite the fact that staining seemed limited by the impermeability of the epidermis, a wide variety of penetrant chemicals ranging from neuramidase to dimethyl sulfoxide failed to enhance staining. The details listed under separative and staining techniques must be followed carefully to achieve uniform consistent results.

Some of the critical sources of error include:

- failure to immerse entire epidermal specimen in fluid.
- failure to keep precisely to time schedule provided. (Specimens must not be held in saline solution or kept overnight.)
- failure to use solutions fresh and clear.
- failure to mount specimen dermal side up.

The area differences in staining are considerable. In the albino guinea pig the external ear, either side, is the best specimen for staining. It exhibits as many as a thousand Langerhans cells per square

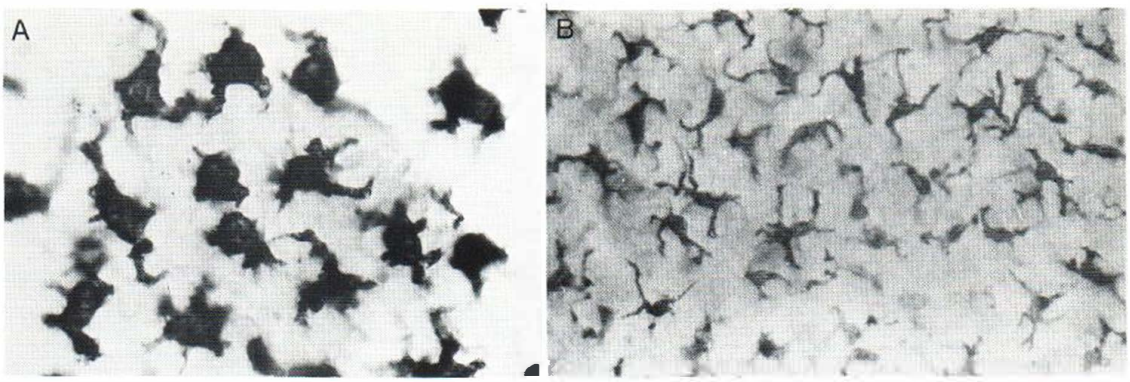

Fig. 3. ATPase stain of Langerhans cells in guinea pig epidermal sheet. (A) separated by pH 11 buffer. Note the cells are swollen, and have short dendrites compared with those seen in recommended technique (Fig. 1 A). ×410. (B) separated by lysozyme technique. Note the cells are smaller and have finer dendrites than those seen in recommended technique (Fig. 1 A). ×410.

millimeter. The hairy areas such as the scalp in man or the body in the guinea pig are notoriously understained. In the case of the foot pad the stratum corneum should be removed prior to initiating any staining procedure.

The morphology and size of the cells varied with the specimen, as well as the method of staining and separation used. The ATPase stain demonstrated the dendritic cells in normal skin but fails under certain experimental conditions such as freezing (Table III). Here the gold stain has an advantage and shows the dark cells sharply. In diseased skin such as contact dermatitis the ATPase method failed to demonstrate the cell dendrites, whereas the gold and paraphenylenediamine stain clearly outlined the dendrites. In some examples of disease states we found staining only with the ATPase method.

Table III. *Langerhans cell staining. Sample results of 30 minutes pretreatment (chemical concentration 1/1 000 in saline) of separated guinea pig epidermis (washed in saline 30 minutes, room temperature)*

Standard method (see text); grading 0 to 4+ (as in Table II)

Pretreatment	ATP	Gold	Paraphenylenediamine	Cobalt
Collagenase	+++	+	+++	+
Ribonuclease	++	++	++	+
48/80	++	0	+++	++
Colchicine	++++	++	++	++
Distilled water	0	++	+++	+++
Freezing −15°C	+	++++	+	0
Saline	++++	+++	++++	++++

In any stain for Langerhans cells, the question of specificity must be asked. Are the cells being stained true Langerhans cells, are they melanocytes, or are they intermediate cells? The most cogent evidence that the cells being stained by the techniques we have described are actually Langerhans or intermediate cells and not melanocytes comes from the cross section studies (Fig. 2). In these sections it can be seen that many of the stained cells are in the mid or upper epidermis. This is in agreement with the usual location of the Langerhans cell and speaks against the cells being melanocytes. Furthermore, dopa studies done by us stain the melanocytes in human epidermis at a level well below the present staining and in a different pattern when viewed surface-wise. In the albino guinea pig, dopa-positive cells were virtually nonexistent.

Not only does the level of the cell network stained by us support the view that these are Langerhans cells, but additional observations are equally convincing. Morphologically, the cells stained by us have a thicker broader dendritic structure than the melanocyte. The number of cells, nearly 1 000/mm² (14), as well as their regular patterned spacing, are typical of the Langerhans cell. Finally, these cells are dopa-negative.

The gold sodium thiomalate stained cells (Figs. 1, 2) pose a special problem of interpretation. The absence of dendrite staining, as well as the selectivity of staining as seen in the combined ATPase–gold technique (Fig. 4), would suggest that the gold

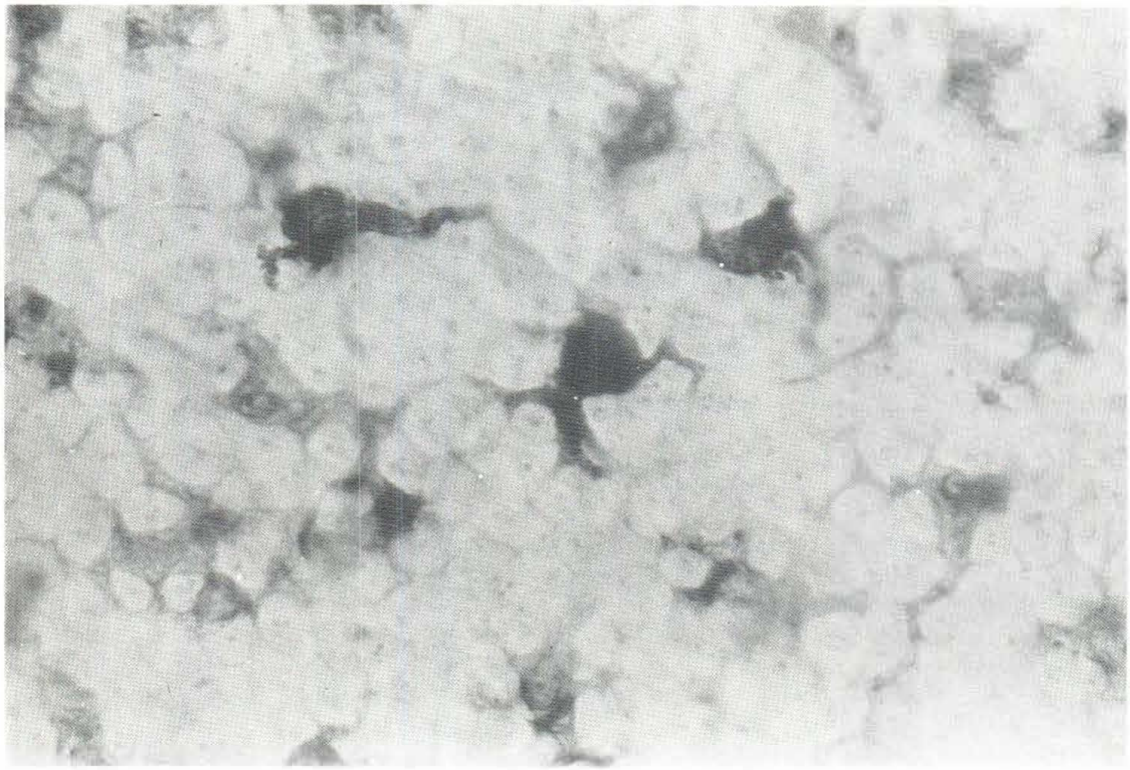

Fig. 4. Combined ATPase and gold stain of vitiligo specimen of human epidermis. Note dark round dendritic cells (stained by gold) and lighter dendritic cells (stained by ATPase). ×785.

might be staining a special type or functional form of the Langerhans cell. Could the gold stain be identifying the intermediate cell?

In regard to the specific stains, we would emphasize our observation that the staining for ATPase was significantly improved by the addition of the energy source, glucose, to the ATP–Pb medium. We can confirm Mackenzie & Squier's observation (6) that EDTA separated epidermis is stained in a superior fashion. Note in Fig. 3 the swollen Langerhans cells with stubby dendritic processes found on staining epidermis separated by means of an alkaline buffer. Interestingly, the use of lysozyme to separate epidermis, first described

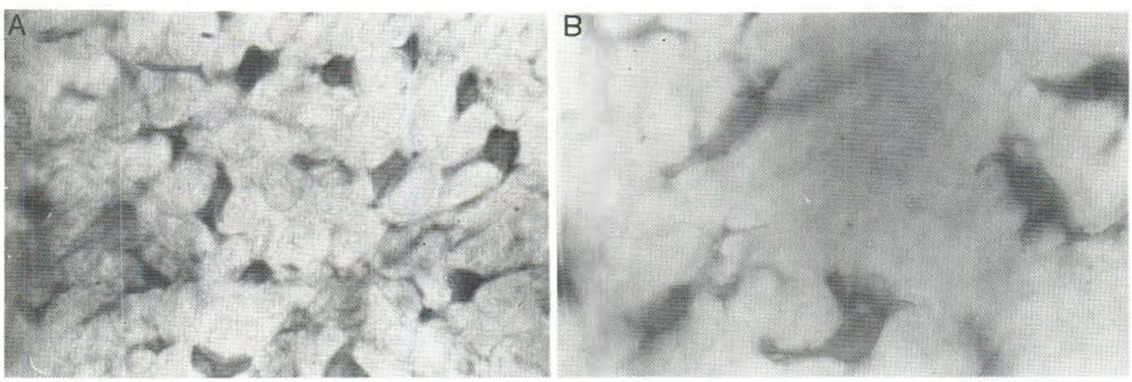

Fig. 5. Paraphenylenediamine stain of epidermis. (A) Guinea pig. ×400. (B) Human. ×770.

herein, was associated with slender Langerhans cells, showing a fine dendritic structure.

Our experience with the classical gold chloride stain was unsatisfactory. In the quest for a better technique we found that gold sodium thiomalate, used so widely in clinical therapy, gave excellent gold staining of the Langerhans cell. It was especially critical that the thiocyanate used for separation be completely washed out before staining was initiated. The use of an acidic pH 3.0 gave extremely sharply and darkly stained, round Langerhans cells. Usually in the guinea pig under these conditions, dendrites were absent. As the staining solution pH was varied and approached pH 7.0, dendrites became evident but the remarkable sharpness of the stain disappeared. In an alkaline medium they did not stain (Table III). At pH 6.4 we found the keratinocytes very darkly stained. The technique described above gave the best results, being especially satisfactory for the staining of inflammatory diseased skin. The use of the classical Timm's solution (12) was valuable as an intensifier of this metallic stain.

The use of paraphenylenediamine as a stain for the Langerhans cell is entirely new. In its most elementary form it served to demonstrate that the Langerhans cell is an allergen trap (10). In the present technique it serves as a reliable means of detecting the cell, although the cell may appear somewhat distorted in the guinea pig epidermis as compared with the ATPase-positive cells (Fig. 5). The use of hydrogen peroxide as a developer is essential for good contrast but must not be used for prolonged periods. Any prolonged exposure leads to overstaining of all cells. Our experience would suggest that the slides should be protected from light. Strangely, some specimens improve on standing—others deteriorate. Thus, generally, early or serial readings are desirable.

The cobalt chloride stain, as well as that of nickel chloride, is new and provides yet another histologic probe for the presence of Langerhans cells. The use of a modified Timm's solution proved essential for the developing of these stains.

The mechanism of staining for ATPase is well known but the new stains we have described are still of an obscure nature. Presumably, membrane enzyme systems are responsible but precise understanding must call for further study.

Although Riley lists some 12 techniques reported to demonstrate the Langerhans cell by light microscopy (9), many of these proved in our hands to be capricious, unreliable and technically difficult. Recently Falck et al. have demonstrated a pronounced L-dopa uptake by the Langerhans cells which could be brilliantly visualized with fluorescent microscopy (4). It is hoped that this and the new battery of stains we have described herein will prove to be more practical, reproducible and successful.

ACKNOWLEDGEMENTS

Technical assistance was provided by Mrs Lynne Todoroff and Miss Inger Pihl, and photographic assistance by Mr Edward Glifort.

Support for this study was provided by Westwood Pharmaceuticals Inc., The Smith Kline Corporation, and the Swedish Medical Research Council.

REFERENCES

1. Baumberger, J. P., Suntzeff, V. & Cowdry, E. V.: Methods for the separation of epidermis from dermis and some physiological and chemical properties of isolated epidermis. J Nat Cancer Inst *2:* 413, 1942.
2. Birbeck, M. S., Breathnach, A. S. & Everall, J. D.: An electron microscopic study of basal melanocyte and high level clear cell (Langerhans cell) in vitiligo. J Invest Dermatol *37:* 51, 1961.
3. Breathnach, A. S. & Wyllie, L. M.-A.: The problem of the Langerhans cells. *In* Advances in Biology of Skin, vol. 8, The Pigmentary System (ed. W. Montagna & F. Hu). Pergamon Press, New York, 1967.
4. Falck, B., Agrup, G., Jacobsson, S., Rorsman, H., Rosengren, E., Sachner, K. & Ogren, M.: Uptake of L-dopa and functionally related aromatic amino acids into the Langerhans cells. J Invest Dermatol *66:* 265, 1976.
5. Lerner, A. B. & Hendee, J. R.: A rapid histochemical test for mammalian tyrosinase. J Invest Dermatol *60:* 16, 1973.
6. Mackenzie, I. C. & Squier, C. A.: Cytochemical identification of ATPase-positive Langerhans cells in EDTA-separated sheets of mouse epidermis. Br J Dermatol *92:* 523, 1975.
7. Mustakallio, K.: Adenosine triphosphatase activity in neural elements of human epidermis. Exp Cell Res *28:* 448, 1962.
8. Niebauer, G.: Dendritic cells of human skin. *In* Experimental Biology and Medicine, vol. 2 (ed. E. Hagen, W. Wechsler & F. Zilliken). S. Karger, Basel, 1968.
9. Riley, P. A.: The Langerhans cell. *In* The Physiology and Pathophysiology of the Skin (ed. A. Jarrett), vol. 3, p. 1199. Academic Press, New York, 1974.

10. Shelley, W. B. & Juhlin, L.: Langerhans cells form a reticuloepithelial trap for external contact antigens. Nature *261:* 46, 1976.
11. Silberberg, I., Baer, R. L. & Rosenthal, S. A.: The role of Langerhans cells in allergic contact hypersensitivity. A review of findings in man and guinea pigs. J Invest Dermatol *66:* 210, 1976.
12. Timm, F.: Zur Histochemie der Schwermetalle Das Sulfid-Silberverfahren. Dtsch Z Gerichtl Med *46:* 706, 1958.
13. Wolff, K.: The Langerhans cell. *In* Current Problems in Dermatology (ed. J. W. H. Mali), vol. 4, p. 79, S. Karger, New York, 1972.
14. Wolff, K. & Winkelmann, R. K.: Qualitative studies on the Langerhans cell population of guinea pig epidermis. J Invest Dermatol *48:* 504, 1967.

Received July 12, 1976

W. B. Shelley, M.D.
Department of Dermatology
University Hospital
3400 Spruce Street
Philadelphia, Pennsylvania 19104
USA